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Number 3

Physiological Zoölogy

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THE UNIVERSITY OF CHICAGO PRESS
CHICAGO, ILLINOIS, U.S.A.

PHYSIOLOGICAL ZOÖLOGY

A Quarterly Journal of Zoölogical Research

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Claims for missing numbers should be made within the month following the regular month of publication. The publishers expect to supply missing numbers free only when losses have been sustained in transit and when the reserve stock will permit.

Business correspondence should be addressed to The University of Chicago Press, Chicago, Ill.

Communications for the editors and manuscripts should be addressed to W. C. Allee, Managing Editor of PHYSIOLOGICAL ZOÖLOGY, The University of Chicago, Chicago, Ill.

The articles in this journal are indexed in the *International Index to Periodicals*, New York, N.Y.

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Entered as second-class matter October 27, 1928, at the post-office at Chicago, Illinois, under the Act of March 3, 1879.

[PRINTED IN U.S.A.]

Physiological Zoölogy

Vol. XI JULY 1938 *No. 3*

MODIFICATIONS OF DEVELOPMENT IN RELATION TO DIFFERENTIAL SUSCEPTIBILITY OF THE BRUCHID (COLEOPTERA) EGG TO KCN DURING DIFFERENT METABOLIC PHASES

(Twenty-one figures)

ALFRED BRAUER
Department of Zoölogy, University of Kentucky

THERE is an abundance of experimental evidence showing that organismic pattern is physiological, rather than morphological, in its earliest manifestation. It has likewise been demonstrated in numerous instances that early physiological patterns are factors in the establishment of the permanent morphological pattern (Child, 1924). Bellamy (1919) has shown that a physiological pattern precedes the development of the morphological pattern in the frog's egg, and that the latter can be modified at will by altering the former.

In the insect the egg at the time of oviposition is not a mosaic of prospective values, as was formerly supposed. It has now been demonstrated for insects of several orders that such values are determined progressively soon after the laying of the egg (Seidel, 1929, 1934; Schnetter, 1934; Brauer and Taylor, 1936).

During this period of determination the pattern of the insect embryo is labile and may be modified by disturbances such as constrictions involving the anterior fourth of the presumptive germ band or cautery of the same area. After these experiments regulation forms, such as complete embryos posterior to the constriction, are common.

For the purpose of analyzing further the determination of pattern in the bruchid egg, especially as regards the determination of the primary axis, it was decided to attempt its experimental modification at various periods by altering physiological processes through their differential susceptibility to KCN. It has been demonstrated (Hyman, 1919) that cyanide depresses the rate of oxygen consumption, and its differential depression by this method is the subject of many papers (see Child, 1924, p. 76).

The specific questions to which answers are sought are: (1) Is there any evidence of physiological pattern in the egg during blastokinesis or during that period when morphological pattern is known to be labile? (2) After formation of the blastoderm, is there any indication of axial pattern before it becomes visibly apparent? (3) If physiological patterns are apparent in the egg, is it possible to produce characteristic modifications of development by their alteration?

It is a pleasure gratefully to extend acknowledgments to Dr. C. M. Child for his interest in the problem and for valuable suggestions which he offered.

METHODS

Gravid female pea weevils were confined to vials containing sterilized peas for 15-minute intervals, after which the peas were replaced by fresh ones. The peas with the newly laid eggs attached were divided into from three to five lots of 6 to 10 eggs each, depending upon the number of eggs available at one time. After incubating the eggs to the desired age at a temperature of 30° C., the different lots were immersed in KCN M/100 for varying periods of time. The process was repeated until a sufficient number of eggs of each stage had been treated to give the experiment value. The minimum number of eggs for a time period of treatment at a particular developmental stage is 14, the maximum number 50, the average 25. Thus for example, several lots of eggs, each with from 6 to 10 eggs, which had been incubated for 2 hours, were treated for various periods of time. One lot was treated for 10 minutes, another for 15 minutes, another for 20 minutes and so on, until egg lots had been treated from 10 to 50 minutes or the lethal period of 100 per cent of the eggs at this stage.

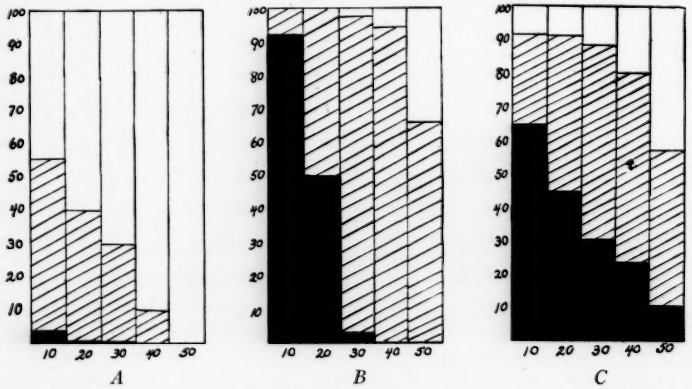


FIG. 1.—Graph showing percentage of complete development (solid bars), teratological development (shaded), and no development (unshaded). *A*, first phase. *B*, second phase. *C*, third phase. Abscissas, time of treatment in minutes. Ordinates, percentages.

After treatment in cyanide the eggs were thoroughly washed, dried on filter paper, and incubated. Those which were to be examined for immediate effects of treatment were returned to the incubator for only 1–2 hours, while those to be examined for teratological development were incubated for 48 hours longer. The eggs were then removed from the peas under dissecting binoculars, fixed, stained *in toto*, and cleared. From this point they were first studied entire, and in many cases drawings were made. They were imbedded by the celloidin-paraffin method. The celloidin blocks containing eggs were often filed away in vials of Kornhauser's clearing mixture. In this they could be re-examined at will, since the blocks were entirely transparent, or they could be imbedded in paraffin for sectioning.

Susceptibility to KCN and Modifications of Development

I. First phase: ½—6½ hours' incubation

A. General susceptibility

Eggs of less than 6½ hours' incubation show almost immediate susceptibility when immersed in the solution, to the extent that they give grossly anomalous development or no organized development after further incubation. After only 10 minutes' treatment, 45 per cent of the eggs give no development; 41 per cent give axiate but highly anomalous development; while only 4 per cent give complete development. The latter

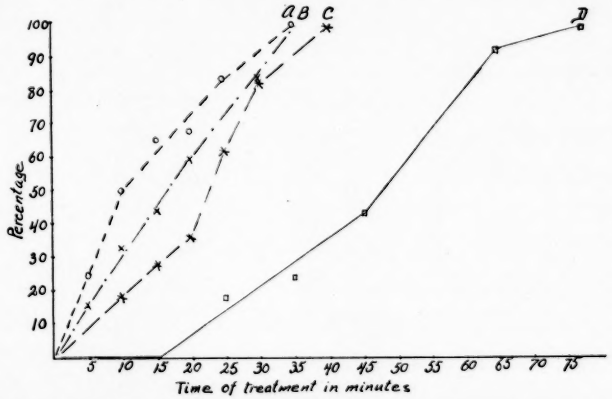


Fig. 2.—Graph showing comparative susceptibility in first two phases. Abscissas give time of treatment in minutes. Ordinates show percentage of eggs killed. *A*, *B*, *C*, treatment during first phase at 1, 2½, and 4½ hours, respectively. *D*, treatment at 7½ hours (second phase) for comparison.

include complete embryos which show microcephaly, and other slight defects of general form. From this point unorganized development increases progressively up to 50 minutes' treatment, after which no eggs were found to give axiate development (Fig. 1*A*).

There is a difference in the initial reaction of eggs to treatment during this interval of time. Eggs treated immediately after oviposition react to treatment more quickly than do eggs 2½ hours old, and the latter more quickly than eggs 4½ hours old. The time required for killing lies between 35 and 40 minutes for any age less than 6½ hours (Fig. 2). Eggs during the first phase, though showing no axial development, frequently give considerable tissue proliferation. It has been found that some eggs, seemingly undergoing no development upon gross examination, nevertheless show some cellular proliferation when sectioned. Any axial development may always be distinguished in stained and cleared eggs.

Developmentally, eggs range from 16 to 200 cleavage nuclei or more during this phase. There is considerable range of difference in any batch of eggs all the same age.

B. DIFFERENTIAL SUSCEPTIBILITY: GROSS MODIFICATIONS

A variety of developmental modifications are exhibited by treated eggs of the first 6½ hours, which, however, are found to fall under one of three types, as follows:

Unorganized terata: In some of these no development whatsoever is apparent in the stained and cleared eggs. Eggs are quite homogeneous throughout. These are uncommon when treated for short periods, and increase in frequency for treatments of longer duration. In greater number are the eggs which contain darkly stained clumps of material which are amorphous and scattered. They are found laterally, anteriorly, or posteriorly. The formless clumps have been identified as unorganized terata.

Axial terata: Of these, there are varied examples. The most anomalous appear much like the darkly stained clumps of the preceding group excepting that they are axiate

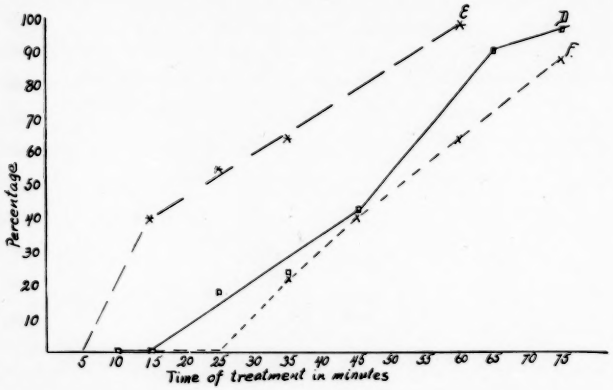


FIG. 3.—Graph showing comparative susceptibility after first phase. *D* and *E*, second and third phases, respectively. *F*, susceptibility after the third phase.

and exhibit segmentation. The anterior portion is sometimes differentiated into cephalic lobes. Others are quite complete embryos but are dwarfed and distorted (Fig. 4).

Complete embryos: These occupy the entire ventral surface of the egg and are differentiated into head, thoracic, and abdominal segments. The embryos are anomalous in that they are microcephalic giants with poor development of head and mouth parts. The bodies are very broad. Segmentation is not distinct, and the ventral chain of ganglia is poorly developed. Terata of this type appear in eggs less severely treated and having a more complete recovery than those of the preceding forms (Fig. 5).

1. *The egg immediately after treatment: cytology.*—For this study, eggs were treated after 2, 4, and 6 hours' incubation. After treatment they were returned to the incubator for only a short time (½–1 hour) or until it was thought that sufficient time had elapsed for any injury to become visibly apparent. They were treated for 25 minutes, or sufficiently long to inhibit development of all but a small percentage of the eggs. They were then removed from the peas, fixed, and sectioned for microscopic examination.

In all such eggs examined, cleavage nuclei were found undergoing mitotic division. No injury appears in the cells which have not reached the egg cortex. They retain their

staining capacity and in all respects appear normal. Nuclei which have reached the cortex but have not entered it, at 4–6 hours, remain under the cortex and do not become engulfed by it in the characteristic reaction exhibited by cleavage nuclei and cortical cytoplasm of normal eggs. The nuclei, however, still appear normal and exhibit mitosis. Nuclei already in the cortical cytoplasm at the time of treatment soon lose their capacity to hold stain, and become shrunken.

The effects of treatment first become noticeable in the oöplasm, and particularly in the cortical cytoplasm. This appears contracted and shrunken, while the oöplasm just under the surface begins to undergo a vacuolation, apparently due to a contraction of the shrunken cytoplasm, which leaves the yolk globules behind in the interstices (see Fig. 13). It is the cortical cytoplasm of the treated eggs which appears to lose its capacity to react with cleavage nuclei arriving at the surface.

In eggs treated prior to 4 hours' incubation, little differential susceptibility is discernible. The cortical cytoplasm generally is very susceptible, and effects of treatment are easily seen in micro sections.

FIG. 4

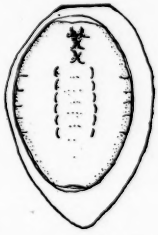


FIG. 5

FIG. 4.—Axiate dwarfed embryo occupying a portion of ventral egg surface (camera lucida).
FIG. 5.—Microcephalic giant of first phase (camera lucida).

2. *The egg after further incubation.*—Unorganized development: In section, eggs of this type are found to contain darkly staining patches of cells lying near the surface in various parts of the egg. These tissue patches appear to be the result of a continued proliferation of cleavage cells after treatment of the egg. The original cleavage cells arriving at the egg cortex, especially in the ventral surface near the anterior border, fail to become incorporated into the severely injured cortical cytoplasm. They continue to proliferate, however, and instead of forming a blastoderm in the cortical cytoplasm, form a clump of cells under the surface. These clumps are syncytia in which cellular boundaries are not distinguishable (Fig. 6).

In these eggs the cortical cytoplasm, as well as much of the internal cytoplasm, shows irreparable injury. On the ventral surface, cortical cytoplasm is usually entirely absent. On the lateral and dorsal surfaces, sometimes short strips remain. The cellular masses always appear to occur where there is some cortical cytoplasm; but in eggs of unorganized development, no blastoderm is formed, however.

The vacuolated areas within the egg are probably due to destruction of internal cytoplasm and subsequent shrinking, causing the yolk globules to mass together in the interstices.

Eggs treated between 4 and 6½ hours' incubation already have a few cleavage cells

imbedded in the cytoplasm of the anteroventral region. After treatment these cells may either fail to proliferate (and merely degenerate) or they may divide without further synthesis of protoplasm and form, over a portion of the surface, a thin, flattened layer containing nuclei. Whether these cells degenerate or whether they form a regulation blastoderm in these areas probably determines whether the subsequent development is unorganized or organized.

If any differences in reaction are apparent, the cortical cytoplasm of the ventral surface near the anterior end shows the first evidence of susceptibility, followed by that of the anterior end and by that of the more posterior ventral surface, while that of the dorsal side is last to show the effects (Fig. 7).

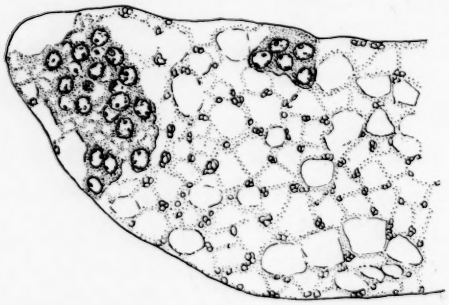


FIG. 6

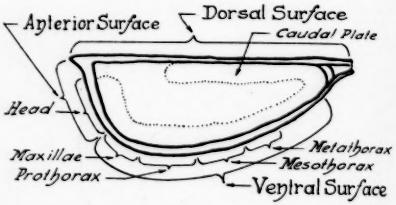


FIG. 7

FIG. 6.—Section of first-phase egg with unorganized development. Note that little cortical cytoplasm remains in egg.

FIG. 7.—Diagram of a medial longitudinal section of an egg showing surfaces and the presumptive regions of the egg surface.

Differential effects of cyanide treatment during the first phase become definitely distinguishable in the cortical cytoplasm of eggs treated after 4 hours' incubation, or during the latter part of the first phase. In these the first and most apparent effects are in the cortical cytoplasm of the ventromedial surface near the anterior border. Here the cortical cytoplasm becomes very thin, and under it vacuoles form in the oöplasm. The effects diminish gradually from this center elliptically in all directions. The dorsal cortical cytoplasm is the last to show injury effects. The egg region showing greatest susceptibility is the region at which cleavage nuclei normally first reach the cortical cytoplasm, and where a short time later the greatest concentration of cleavage cells occurs (Fig. 8).

When an egg treated at $4\frac{1}{2}$ hours and incubated $2\frac{1}{2}$ hours longer is superimposed over one not so severely treated and which has undergone further development, the injured areas of the two show fairly close coincidence. The affected area in the developed embryo extends from the prothoracic region up through the region of the mouth parts (Fig. 9).

Organized axial terata: In this group are eggs containing embryos which range from small darkly staining dwarfed terata to large, more complete embryos with fairly well-formed segments. All of these embryos have cephalic lobes and some body segmentation.

The more poorly formed lie far laterally in the egg, usually in the angle between the convex ventral surface and the flattened dorsal surface. The head lobes are small, and the body segments are often asymmetrical. In others, head lobes are quite well developed. The body segments appear well formed but are highly anomalous.

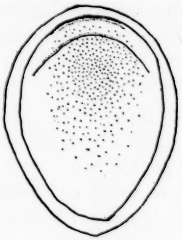


FIG. 8

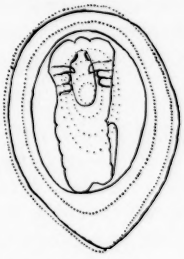


FIG. 9

FIG. 8.—Diagram of ventral view of an egg showing differential susceptibility to KCN. Densely stippled area shows greatest susceptibility.

FIG. 9.—Outline drawing of a first-phase egg with embryo, showing deficient area from prothoracic region through maxillary region (solid lines), superimposed over a first-phase egg (stippled outline) fixed 2 hours after treatment. Stippled egg shows region of ventral surface most affected by treatment (inner eliptical stippled lines). Outline by camera lucida.

Sections show the anterior end of these embryos to be histologically differentiated. The ectoderm of the head lobes shows severe injury in that the cells are very irregular in form and outline, with nuclei of varying size. Head segments contain an entodermal canal with the cells of this germ layer considerably better developed than the ectoderm.

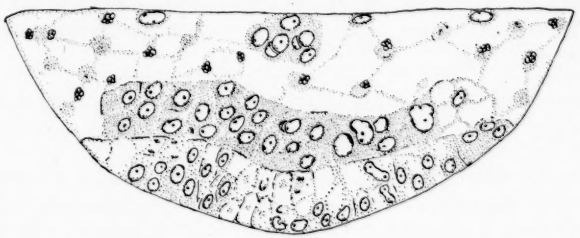


FIG. 10.—Section of organized embryo of first phase. Injury is most apparent in ectoderm. Development of mesoderm is abnormally great.

Posterior to the head region the cortical cytoplasm appears to have been injured considerably more severely. Here ectoderm, normally the most prominent layer, is present, though poorly formed. Its cells are irregular or flattened. There is much evidence of cellular disintegration and occasional evidence in some parts of mitotic activity on the part of nuclei. The mesodermal layer is thicker and usually more abundant than in the normal embryo (Fig. 10). Farther posteriorly, development of ectoderm is less complete and mesoderm becomes reduced, though there are differences in individual embryos.

The outstanding difference between the organized terata of this series and the unorganized formations of the former is that in all of the organized embryos an ectoderm is present. This undoubtedly means that the cortical cytoplasm of the egg was less severely injured than in the former series or that recovery took place, so that cleavage cells reacting with this cytoplasm formed ectoderm. The ectoderm of these embryos is undoubtedly responsible for the formation of the inner germ layers by the mitotic division of ectodermal nuclei, though in some embryos the evidence for this is scanty.

C. ANALYSIS OF MODIFICATION IN FIRST PHASE

The entire period of development from $\frac{1}{2}$ hour to $6\frac{1}{2}$ hours after laying is highly susceptible to KCN. Treatment of eggs for short periods results in grossly anomalous or in unorganized development if it is not prevented altogether.

Unorganized development is the result of a proliferation of cleavage cells within the egg, which have failed to become incorporated in the cortical cytoplasm.

Axial development is accomplished after light dosage in some cases. Such embryos are formed by the germ layers. Ectoderm is formed where cleavage cells have been incorporated into the cortical cytoplasm of the egg. It shows severe effects of KCN treatment. Mesoderm, formed from the injured superficial cells, shows little cytological injury.

Injury effects of treated eggs are apparent in the cortical cytoplasm and in the internal cytoplasm of the egg. They appear to be greatest on the ventral surface near the anterior part of the egg. Because of this injury of cortical cytoplasm, cleavage cells fail to become imbedded in it, and no blastoderm is formed. The cleavage cells which have not arrived at the egg periphery, on the other hand, continue to divide.

When cleavage cells become incorporated in the cortical cytoplasm, a continued development results in an axiate embryo. The more perfect of these lie on the ventral surface, while the dwarfed and badly deformed ones usually lie far laterally. Conversely, all cellular proliferation within the egg, away from the cortex, results in unorganized cell masses.

II. SECOND PHASE: $6\frac{1}{2}$–12 HOURS

A. GENERAL SUSCEPTIBILITY

Developmentally this is the period in which the blastoderm is formed. All of the cleavage cells reach the cortical cytoplasm during the early part of the period; and by the latter part (12 hours) the cells on the ventral surface become crowded and columnar, to form the ventral plate. Visible axial development of this plate is then apparent.

During the early part of this phase, although the eggs are still undergoing blastokinesis and are visibly but slightly advanced over the eggs of the latter part of the first phase, they show a markedly different reaction to treatment with KCN.

In the first place the eggs are more resistant. This is shown, first, in that a longer period of treatment is required before they show evidences of treatment and, second, in that a much greater dosage is required to inhibit any further development (see Figs. 1*B* and 3). More than 15 minutes of treatment is required before any of the eggs show effects. From this time the curve rises somewhat slowly to a dosage of 45 minutes, then more rapidly to 60 minutes, after which 90 per cent of the eggs are killed. Seventy-five minutes of treatment is required to prevent any of the eggs from showing further de-

velopment. Even after the effects of treatment become apparent, these are in contrast to those of the first phase in that all of the treated eggs show an axiate, organized development, though anomalous.

In the second place this phase is distinguished from the first by the type of terata produced. All of these are axiate, as mentioned above. The terata are elongate, twisted, and distorted. Their segmentation is deep and sometimes appears bilaterally asymmetrical. Cephalic lobes are well formed, and rudiments of mouth parts are present. The ventral ganglionic chain is apparent in some (Fig. 11).

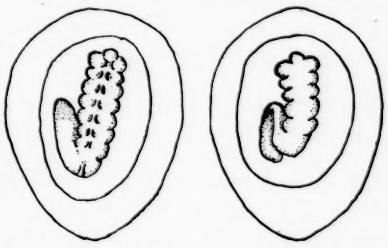


FIG. 11.—Terata of the second phase

For an understanding of this anomaly it is necessary to refer to normal development of the beetle embryo during the period of germ-layer formation. During this period the ventral plate, which has formed on the ventral egg surface by a thickening of the blastodermal cells, elongates posteriorly and flexes around the posterior extremity of the oöplasm and pushes dorsally and anteriorly until a caudal extension is formed which almost touches the cephalic lobes forming at the anterior extremity of the plate. The ventromedial cells of the ventral plate then become very tall, undergo mitotic nuclear division, and form the mesodermal layer internal to the ectodermal cells of the ventral plate.

The terata of the second phase are maldevelopments of the elongation of the ventral plate. Instead of flexing normally dorsoanteriorly and slightly to the right, they may flex in any direction. Since the plate is limited from extending farther posteriorly by the confines of the egg, it becomes displaced and distorted, twisted and turned. The normal dorsoanterior flexion appears to be inhibited, as few embryos succeed in completing this phase of development.

B. DIFFERENTIAL SUSCEPTIBILITY: HISTOLOGICAL

1. *Eggs incubated after treatment.*—Only one type of egg need be described here, since treated eggs capable of further development develop as organized axiate embryos.

In section the ventral plate of these embryos is usually narrower laterally than the normal, and is thinner vertically. There can be little doubt that the primary injury is to the ventral ectodermal plate. In the more severely injured, only a few small scattered nuclei remain. Cell outlines are obliterated, and occasional empty vacuoles are found. Strangely, the shape of the normal ectodermal layer is retained. This is thicker in the center and thinner laterally.

The mesodermal layer, normally a thin sheet of cells lying over the inner surface of the ectoderm, is here abnormally thick and abundant. It not only covers the inner surface of the ectoderm but extends farther laterally than the ectoderm. This layer is a cellular syncytium (Fig. 12).

The section illustrated also passes transversely through the tip of the caudal plate. Here no ectoderm is found, although the mesodermal mass appears to retain lateral prominences and a ventral groove. Mesoderm of the caudal plate is also shown to extend farther laterally than normal. This regulation embryo is largely a mesodermal embryo.

An egg showing extreme displacement of the embryonic plate laterally is shown in Figure 13. The ectoderm of this egg has shown a greater recovery than that of the former, and a prominent medial groove is present. Cellular debris is found in the ventral

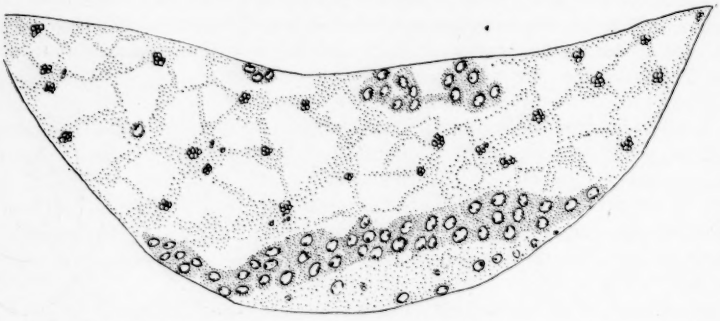


FIG. 12.—Section through thoracic portion of second-phase embryo. Tip of caudal plate shown on dorsal (concave) side of egg. Ectoderm almost absent in this region. Mesoderm hyperplastic.

groove, indicating that the medial portion of the plate was either more severely injured or that it did not undergo recovery. The organization of ectodermal cells into two lateral masses indicates an attempt at formation of a ventral chain ganglion.

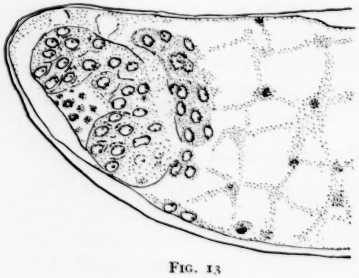


FIG. 13

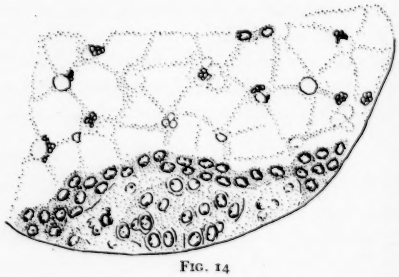


FIG. 14

FIG. 13.—Section of second-phase embryo in abnormal position. Ectoderm shows considerable recovery. Mesoderm hyperplastic.

FIG. 14.—Section of mid-thoracic region of second-phase embryo showing differential recovery in the ectoderm. Development in center of ectodermal layer is the first rudiment of the central nervous system, making a delayed appearance.

Mesodermal cells are abundant but at this level do not lie so closely applied to the ectodermal cells. The egg likewise shows extreme injury to the cytoplasmic cortex, and this injury becomes more pronounced medioventrally.

Differential recovery is illustrated in the ectoderm of some embryos (Fig. 14). The ectodermal cells of the embryonic plate are distinct medially and have formed a central ganglion-like structure which is in the first stages of development. Lateral to this the ectodermal cells are still very indefinite. Mesodermal cells have spread laterally beyond

the limits of the ectoderm. Mesoderm is again much more abundant than it is in the normal embryo.

2. *Eggs not incubated after treatment.*—In eggs which are fixed, stained, and cleared just after treatment, the cleavage cells are seen at the egg surface. These are normally more numerous on the ventral surface just behind the point of the egg having the greatest vertical diameter. This is also the region at which the cleavage cells first reach the cortical cytoplasm. From here the concentration of cells diminishes over the remainder of the surface to the dorsal cytoplasm, where their concentration is least.

In eggs treated at 7 hours and fixed ½ hour after treatment is discontinued, injury is not so distinctly apparent as in those treated in the first phase. However, an area appears at the cell concentration center, where the cells appear less distinct than they do in normal eggs. This area extends forward into the presumptive cephalic lobe level and posteriorly along the medioventral line through the upper thoracic region. The entire area most affected is concentrated more along the median line than it is in first-phase eggs (Fig. 15).

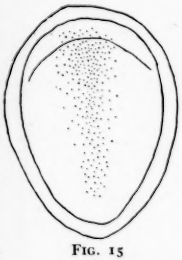


FIG. 15

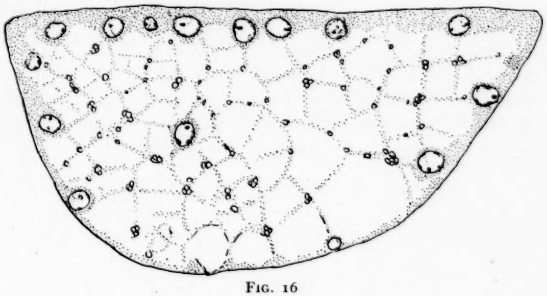


FIG. 16

FIG. 15.—Diagram of an egg of second phase showing differential susceptibility. Egg viewed from ventral surface.

FIG. 16.—Section through presumptive prothoracic level of an egg treated at 7½ hours. The differential susceptibility is shown in the medioventral blastoderm.

A study of sectioned eggs discloses the reason for the differences. Cleavage cells are well implanted in the cortical cytoplasm laterally and dorsally, but ventroanteriorly, where normally they are most numerous and best established, there are relatively few.

Coincident with the scarcity of normal cleavage cells there is a scanty cortical cytoplasm. In many eggs there are spots where it is almost, if not entirely, absent, and vacuoles appear under its former surface (Fig. 16).

Nearer the posterior egg extremity more cleavage cells appear in the cortex, but these are small and are scantily surrounded by cytoplasm. Indeed, the cytoplasm shows signs of great injury. It is the cytoplasm in all cases which appears to have been injured most by cyanide exposure, since nuclei with very little cytoplasm may be found in mitotic division.

C. SUMMARY AND ANALYSIS OF TREATMENT DURING SECOND PHASE

The second phase begins at 6½ hours and lasts until 12 hours at incubation temperature. It is recognized by the increased tolerance of the eggs to cyanide treatment and by the type of terata obtained after such treatment.

All terata of this phase are axiate embryos. They are elongate and abnormally twisted.

The embryos are composed of the germ layers. Of these the ectoderm shows effects of severe injury, but also considerable recovery. Recovery, like injury, is differential within the embryonic plate, namely, in a mediolateral direction, and anteriorly and posteriorly from the region of highest susceptibilty. The internal layer or mesoderm is abnormally abundant and appears to be the dominant tissue of second-phase embryos. It is formed, as in normal embryos, by the mitotic division of ectodermal cells, where the latter have survived.

The type of anomaly is recognizable as a maldevelopment of a particular stage of normal development in which the embryonic plate grows in length. In the terata this occurs abnormally, owing to an excessive amount of mesoderm and inhibition in the injured ectoderm. The embryos become abnormally twisted during elongation of the plate and remain in this condition because the normal contraction fails to follow.

III. THIRD PHASE: 12–16 HOURS

A. GENERAL SUSCEPTIBILITY

In normal development this period is characterized by the thickening of the ventral blastoderm and the growth in length of the ventral plate thus formed. The plate flexes dorsoanteriorly at its posterior extremity and forms the caudal plate. Ventromedially

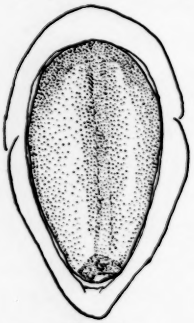
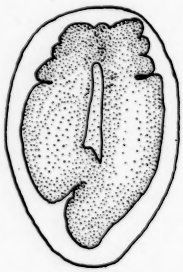
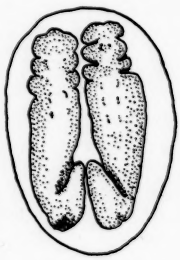


FIG. 17



A



B

FIG. 18

FIG. 17.—Egg of 16½ hours, showing thickening of ventral plate as a dark medial band. The thickening is due to the formation of the mesentodermal layer.

FIG. 18.—*A*, embryo of third phase, showing partial duplicity. Doubling begins in prothoracic region and extends anteriorly into the head and posteriorly through the lower thorax. *B*, twinning or complete separation and reduplication of bilateral parts.

the columnar cells of the blastoderm become tall. By their mitotic division the mesodermal layer is formed as a thin lamina of cells lining the embryonic plate. At the anterior extremity of the plate, and likewise at the extremity of the caudal plate, this internal layer becomes the entodermal rudiment. In the more advanced embryos of this stage the ectodermal cells of the medial portion of the embryonic plate of the thoracic region are proliferating to form the anlagen of the ventral ganglia. Posteriorly, development is less advanced, and here the ventral plate is still in the process of forming the mesodermal layer (Fig. 17).

During the third phase, eggs are more susceptible to KCN than they are in the second phase, and somewhat less so than they are in the first. Complete inhibition of development is accomplished in 90 per cent of the eggs after from 50 minutes to 1 hour of treatment (see Figs. 1*C* and 3).

Eggs treated for 20–40 minutes tend to produce a doubling of the ventral plate along the anteroposterior axis. This is the characteristic anomaly resulting from treatment at this time, and has been encountered in some form in 80 per cent of some treated batches of eggs.

Doubling (*duplicitas*) in these cases begins in and usually affects only the thoracic portion of the plate. In several cases it extends anteriorly to include the head, while in a few it likewise extends posteriorly and produces complete separation or twinning (Figs. 18*A* and *B*).

FIG. 19.—Anterior and partial posterior duplicity in late third-phase embryo. The prothoracic region, being precocious over other levels, has passed the labile stage and did not divide as did more anterior and posterior levels.

A more complicated form of doubling is illustrated by the embryo shown in Figure 19. It represents *duplicitas anterior* and partial *duplicitas posterior* but is unpaired in the prothoracic region. This egg was treated at 15 hours and was therefore nearing the end of the phase. Determination of definite laterality had probably been realized in the prothoracic region at the time of treatment, and was therefore no longer subject to the same differential susceptibility as the remainder of the plate. Farther anteriorly in the head and farther posteriorly the levels were still labile, and were consequently affected in the characteristic manner. The lower thoracic level and the head level therefore show doubling, while the prothorax is undivided.

B. DIFFERENTIAL SUSCEPTIBILITY

Injury from the effects of cyanide treatment is apparent in the sections of embryos treated for 25 minutes. Again this occurs in the medial portion of the ventral plate, in that region where the first concentration of cleavage cells occurred and which leads in general development. From this area the effects spread anteriorly and posteriorly but

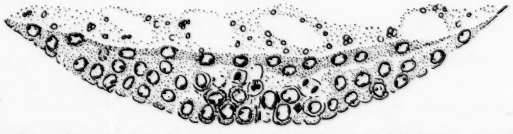


FIG. 20.—Section through anterior thoracic level of treated third-phase embryo. Most apparent injury in medial ectodermal cells.

remain concentrated along the medial portion of the plate. The particular cells most affected are those which are proliferating the ganglionic rudiments of the ventral chain of ganglia obliquely laterad from the median line. Many of the more lateral cells of the plate continue their activity, while the more medial ones become broken down. The impression obtained from this picture is that the center integrating these two slightly lateral areas is destroyed, permitting these regions to continue or resume development without this integration. The most noticeable effects are toward the anterior portions of the embryo, while the caudal plate is the last to suffer (Figs. 20 and 21).

C. ANALYSIS

At the time of treatment (12–16 hours) the ventral plate is in the process of extension and elongation. In the most advanced embryos the central region of the ventral plate is showing the organization of paired centers, the ventral ganglia. Injury is then greatest where regional activity is highest, namely, in the medial portion of the ventral plate. When recovery occurs, centers just lateral to the median line become most active and develop independently of each other. A portion of the embryonic plate becomes organized around each of these centers and produces bifidity.

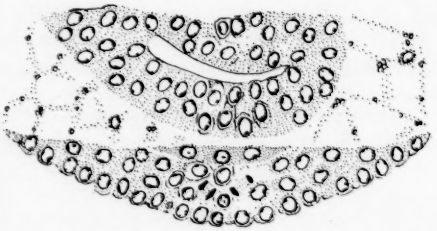


FIG. 21.—Same embryo as Figure 17. Section taken more posteriorly so as to include the basal portion of caudal plate. Injury is apparent in medial portion of ventral plate but is not noticeable in the caudal plate.

As in former phases, the prothoracic region is most highly susceptible. Doubling starts there and extends into the head and into the mesothorax secondarily. The prothorax likewise becomes determined bilaterally in advance of head and body levels.

DISCUSSION

Two evident features of this study are (1) that there are distinct phases during early development in which the reactions of eggs to cyanide treatment differ markedly, and (2) that characteristically distinct developmental modifications are produced in each phase by differential susceptibility of the egg to cyanide, indicative of physiological changes leading to the establishment of a morphological pattern.

SUSCEPTIBILITY PHASES

The distinct changes in susceptibility which occur during early development in *Bruchus*, as shown by Figures 1–3, do not coincide with visible steps in morphogenesis. It is probable that they do mark physiological processes initiating, and to some degree accompanying, developmental processes. For example, the first phase is found to coincide definitely with the period of embryonic determination in this insect (Brauer and Taylor, 1936). This is a physiological phase not delimited by distinct morphogenetic events. Each of the other phases probably also marks regional physiological differences which inaugurate morphogenetic processes.

In this connection it might prove of value to analyze groups of eggs by Bodine's (1929) method of measuring oxygen consumption in insect eggs, to determine whether respiratory differences actually exist during the phases indicated. Bodine (1934) found no significant differences in oxygen intake in grasshopper eggs during active development.

MODIFIED DEVELOPMENT RESULTING FROM DIFFERENTIAL SUSCEPTIBILITY

Bellamy (1919) showed that modifications produced by differential susceptibility of the frog's egg were the result of alterations produced in the physiological egg pattern. As the physiological pattern undergoes progressive developmental changes, cyanide treatment at different periods will be reflected by the different morphological patterns produced.

The same explanations account for the modifications obtained in the Bruchid egg. During the first 6½ hours of development, when the egg passes from an indeterminate to a determinate stage (Brauer and Taylor, 1936), with the exception of an anteroposterior polarity, pattern has not been entirely fixed. In Brauer and Taylor's paper, lability was indicated by the large number of double embryos and by the regulation embryos formed in the posterior two-thirds of the egg alone. In the present paper it is shown by the fact that, after only short periods of treatment, axial development is prevented in a large percentage of cases. Furthermore, this is the only period when cyanide treatment interferes with axiation.

The point is further emphasized herewith that the "lay" of the embryo becomes definitely established during the first phase. During this time the ventral region nearest the anterior surface, namely, the presumptive prothoracic-maxillary region, becomes the area of the first and most decided response to treatment. In this area cleavage cells first reach the cortical cytoplasm, and here they become most concentrated. It leads generally in morphogenesis. Differential susceptibility of this region may change the lay of the embryo laterally, although it maintains its general anteroposterior orientation.

The region of highest susceptibility in the bruchid egg corresponds rather closely to that of the libellulid egg designated by Seidel (1934) as the differentiation center. This conclusion was reached on the basis of constriction, and ultra-violet radiation experiments. Schnetter (1934), on the basis of constriction experiments on the honeybee egg, determined the organization center to be prothoracic-maxillary in location.

The cyanide experiments indicate a bearing on the integrating capacity of the region in the following manner. In eggs treated from 10 to 25 minutes, axial development is frequently inhibited; but in many instances there is still some regulative development of the egg. Such regulation embryos, instead of lying on the ventral surface, are centered around the anterolateral or anterodorsal cytoplasm. Thus, with the disintegration of anteroventral cortical cytoplasm, that of an adjacent region becomes dominant and asserts its more limited capacity to organize. The organizing capacity, like the gradient of differential susceptibility in this cytoplasm, is from the ventral toward the dorsal surface.

By the beginning of the second phase the axiate pattern and the general position of the embryo have become determined. Further alterations are now brought about by differential susceptibility and differential recoveries within this general pattern. The peculiar form assumed by these embryos is due to a modification of the relationships of the primary layers. The abnormal twisting which the embryos undergo is explainable on a mechanical basis, namely, that the inner mesoderm becomes more abundant and more active than the more affected ectoderm. The result of this is that normal flexure of the embryonic plate is prevented— and flexures may even be reversed.

By the modifications produced in the third phase it becomes evident that all parts of the embryonic plate are not yet irrevocably determined, at least as regards bilater-

ality. The modifications produced in the plate at this time range from partial doubling (*duplicitas cruciata*) to complete twinning. This implies that any or all levels of one side of the plate still have the capacity to reduplicate the other side. The reduplication as here produced is explainable in the sections of the embryonic plate. As shown, it is due to the greater susceptibility of the medial portion of the plate, and a subsequent physiological isolation of the more lateral portions, which assume a physiological dominance. Doubling begins at the most susceptible region, which is prothoracic. From here it extends forward into the head and posteriorly through the thorax. It may likewise spread farther anteriorly and posteriorly to produce complete separation and reduplication.

Differential susceptibility has here produced the same effects, after a fashion, obtained by Krause (1934) by mechanical means. By the removal of portions of the embryonic plate of the cricket, *Tachycines*, by puncture, or by incisions made on the plate with a glass needle, he produced various forms of reduplication. He found the type to be dependent upon the part of the plate from which removal of material was made, and upon the stage of development of the embryo. According to his figures, the embryonic plate of *Bruchus* is at approximately the same stage of development at 12–16 hours as that of *Tachycines* at the time the operations were made.

Krause has explained doubling by the mechanical separation of parts and the capacity of these parts to reduplicate because of the regulative capacities retained by the parts up to that particular stage of development. In *Bruchus* it is apparent that the physiological isolation produced by differential inhibition of oxygen intake produces the same effects that mechanical separation produce. The two insects appear to be in close agreement as to the progress of embryonic segregation within the embryonic plate, namely, from prothorax anteriorly and posteriorly.

It will be noted that doubling of the embryonic plate takes place at a different stage than it did after anterior cautery and anterior constriction in former experiments (Brauer and Taylor, 1936). At that time reduplication was obtained during the first phase. The duplicate embryos were always formed at more posterior levels than in the normal egg. In the present cases the embryos retain their natural level, as determined in earlier stages.

DIFFERENTIAL SUSCEPTIBILITY OF EMBRYONIC LAYERS

Certain impressions pertaining to origin of germ layers and their relative values may be obtained in these studies. It must again be pointed out that cyanide treatment during the first phase takes place prior to the arrival of cleavage cells in the superficial cortical cytoplasm. In these eggs it appears that the cleavage cells are less susceptible to treatment than the cortical cytoplasm. The cells continue to proliferate except after very severe treatment, but they do not react with the cortical cytoplasm. The result is that no blastoderm is formed. The masses of cleavage cells without a cortical cytoplasm are incapable of giving any kind of organized development under these conditions. It is thereby indicated that activity of cortical cytoplasm is necessary for the determination of prospective values; and furthermore, when the differential susceptibility of egg regions is taken into consideration, it appears to be the cytoplasm of the anteroventral region that is necessary for complete organized development.

In the second phase the eggs are subjected to treatment prior to the formation of a blastoderm, or, at any rate, before it has been completed. In eggs which continue to

develop after treatment, an ectoderm is always formed—at least in part. Its cells quickly give rise to an internal layer which is the mesoderm, except in two definite areas, where it has been designated as entoderm. The inner layer, either because of its release from the physiological dominance of the injured ectoderm or because it actually absorbs some material from its injured cells, undergoes a hyperplasia. Some of the resulting embryos are preponderantly mesodermal embryos.

THE HEAD

It becomes readily apparent that in the sequence of physiological development of pattern in this insect the head is secondary in origin and remains so during the phases studied. From the works of Seidel, Schnetter, and Krause it is probable that this is the case in other insects. To what factors in development does thoracic dominance owe its origin? For how long during development is this dominance maintained? Surely the insect with its specialized anterior sensory development has a cephalic integrating center! These and other questions arise in this connection, but so far are only suggested.

SUMMARY

1. The development of pattern was studied in the egg of the cowpea weevil by an analysis of the modifications produced by the differential susceptibility of the egg to KCN.

2. In early development three metabolic phases are distinguishable. They are characterized by the degree of susceptibility and by the nature of the modifications produced.

3. The phases are as follows: First phase, from oviposition to 6½ hours after oviposition, during which time the eggs are highly susceptible. The terata of this phase are typically inaxiate. Second phase, from 6½ to 12 hours, during which time the eggs are much more resistant and the terata are elongate and always axiate when development takes place at all. Third phase, 12–16 hours, at which time the eggs are again less resistant than in the second period. The characteristic modification is partial to complete doubling along the anteroposterior axis.

4. During the first phase susceptibility becomes highest on the ventral surface. The most susceptible area gradually becomes restricted to the presumptive prothoracic-maxillary region.

5. Severe injury to the anteroventral cortical cytoplasm prevents its reaction with cleavage nuclei and their aggregation at that place, although the nuclei may continue to multiply within the oöplasm for some time. With destruction of anteroventral cytoplasm a modified embryonic plate may be formed around a new center or centers lying more dorsal. Capacity to form an organized axiate plate appears to become less the farther the new center is removed from the ventral surface.

6. Aggregations of cells are frequently formed by cleavage nuclei, which fail to become imbedded in cortical cytoplasm. These cells may undergo hyperplasia to form amorphic masses.

7. In second-phase eggs injury is most severe in the ectoderm of the embryonic plate. From the presumptive prothoracic-maxillary region injury extends in a declining gradient through the anterior portion of the head and posteriorly through the embryonic plate. Differential recovery frequently follows the same course.

8. Mesoderm (mesentoderm) is always formed in second-phase embryos. With the injury of ectoderm the mesoderm becomes hyperplastic. Its great increase is probably the cause of the abnormal flexions in these embryos.

9. Third-phase eggs are treated in the ventral-plate stage. Injury is severe in the medial portion of the plate anteriorly, and declines anteriorly and posteriorly. Differential recovery follows the same course.

10. Separation of the plate along the anteroposterior axis is easily accomplished by differential susceptibility. When this occurs, there is a reduplication of the other half, by each side. This is an indication that determination of definite bilaterality has not been accomplished at this particular time.

11. The prothoracic region is the first to become definitely determined as to bilaterality. After this occurs, doubling and reduplication are no longer possible in it, although head levels and lower body levels may still reduplicate.

12. The location of the integration center in *Bruchus* agrees with the location of the center in other insects which have been studied, but differs from other axiate animals in that it is not anterior in the head.

LITERATURE CITED

BELLAMY, A. W. 1919. Differential susceptibility as a basis for modification and control of early development in the frog. Biol. Bull., **37**:312–61.

BODINE, J. H. 1929. Factors influencing the respiratory metabolism of a developing egg (Orthoptera). Physiol. Zoöl., **2**:459–82.

———. 1934. The effect of cyanide on the oxygen consumption of normal and blocked embryonic cells (Orthoptera). Jour. Cell. and Comp. Physiol., **4**:397–404.

BRAUER, ALFRED, and TAYLOR, ALBERT CECIL. 1936. Experiments to determine the time and method of organization in bruchid (Coleoptera) eggs. Jour. Exper. Zoöl., **73**:127–51.

CHILD, C. M. 1924. Physiological foundations of behavior. New York: Henry Holt & Co.

HYMAN, L. 1919. On the action of certain substances on oxygen consumption. II. Action of potassium cyanide on planaria. Amer. Jour. Physiol., **48**:340–71.

KRAUSE, GERHARD. 1934. Analyse erster Differenzierungsprozesse im Keim der Gewächshausheuschrecke durch künstlich erzeugte Zwillings-Doppel- und Mehrfachbildungen. Roux' Arch. Entw'mech., **131**:285–323.

SCHNETTER, MARTIN. 1934. Physiologische Untersuchungen über das Differenzierungszentrum in der Embryonalentwicklung der Honigbiene. Roux' Arch. Entw'mech., **131**:285–323.

SEIDEL, FRIEDRICH. 1929. Untersuchungen über das Bildungsprinzip der Keimanlage im Ei der Libelle Platycnemis pennipes. Roux' Arch. Entw'mech., **119**:322–440.

———. 1934. Das Differenzierungszentrum im Libellenkeim. I. Die dynamischen Voraussetzungen der Determination und Regulation. *Ibid.*, **131**:135–87.

———. 1935. Der Anlagenplan im Libellenei. *Ibid.*, **132**:671–751.

ULTRA-VIOLET RADIATION OF GRASSHOPPER EGGS (ORTHOPTERA)[1]

(Seven figures)

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THE numerous contributions in the field of the biological action of ultra-violet light may be divided into four general groups based on the experimental materials used: first, the effects on protoplasm as studied in protozoa and plant cells; second, the effects on marine eggs; third, the bactericidal effect; fourth, the effects on chemical compounds of especial biological importance (Heilbrunn, 1932; Heilbrunn and Daugherty, 1933; Black, 1936; Lillie and Baskervill, 1922; Wyckoff, 1931; Crowe, 1936; Hollaender and Duggar, 1936; Harris, 1926; Spiegel-Adolf and Oshima, 1929; Laurens, 1928; Clark, 1922; Burge, Fischer, and Neill, 1916).

In the present work a quantitative study has been made of the effects of ultra-violet irradiation of the developing eggs of the grasshopper, *Melanoplus differentialis*. This material is especially suitable for use in an investigation of this kind since the course of normal development is well understood (Slifer, 1932*a*) and various physiological considerations are at hand (Bodine, 1929, 1932; Bodine and Boell, 1934; Slifer, 1932*b*).

MATERIAL AND METHODS

The effects of ultra-violet light have been determined largely through changes in the oxygen consumption of the eggs. For this work, modified Krogh differential manometers were employed (Bodine, 1929). Five to ten eggs were used in each manometer, and experiments were performed in such manner that different eggs were used daily as well as the same eggs repeatedly. One hundred or more eggs were used in obtaining data for each oxygen-consumption curve, and from two hundred to one thousand eggs constituted an experiment. Hatching records were also kept.

As a source of ultra-violet light the entire spectrum of an air-cooled quartz mercury-vapor lamp operating on 110–15 volts A.C. and 3.7 amperes at a distance of 15 cm. was used. The eggs were radiated on damp filter paper, in glass dishes covered with quartz plates $2\frac{1}{2}$ mm. thick, or with glass plates 1 mm. thick for experimental and control experiments, respectively. The dishes were placed on wet toweling, and their temperature was kept at 22°–24° C. during the irradiation. The dosage was controlled by varying the length of exposure.

Removal of the chorion was accomplished by placing the eggs on adhesive tape, loosening the chorion with a needle, and then removing the egg, leaving the chorion behind on the tape.

RESULTS

1. *The permeability of egg membranes to ultra-violet light.*—The grasshopper embryo and yolk are surrounded by two membranes during the greater part of embryonic development (Slifer, 1932*a*). When the egg is laid, only the outer membrane, the chorion,

[1] Aided by a grant from the Rockefeller Foundation for work on the physiology of the normal cell.

is present. About the seventh day of development the inner transparent cuticle is laid down, after completion of which the outer membrane (chorion) may be peeled off, permitting direct observation of the embryo. It was first necessary to determine the permeability of these membranes to ultra-violet light.

To test for the penetration of primary radiations, a lithopone paste (Ellis and Wells, 1925) was put between two quartz plates; and pieces of the membranes, moist and dry, were placed on the plates and exposed to ultra-violet light for various intervals up to 2 hours. During this time there was no darkening of the lithopone paste beneath the

TABLE 1

EFFECT OF VARIED AMOUNTS OF ULTRA-VIOLET RADIATION ON GRASSHOPPER EGGS WITH CHORION AND CUTICLE AND WITH CHORION ALONE

Prediapause eggs have only the chorion for 7 days after laying; beyond that time both membranes are present. The values are given in percentage of eggs started and the days after diapause on which the eggs hatch are indicated.

Time of Radiation		Days Postdiapause on Which Eggs Hatch							
		17	18	19	20	21	22	23	Total
Prediapause eggs:									
One hour daily for 5 days from laying	Control	0	0	80	2	6	0	1	89
	Experimental	0	3	88	1	0	1	0	93
One hour daily for 10 days from laying	Control	0	2	80	5	1	0	0	88
	Experimental	0	3	78	0	1	1	2	85
One hour daily for 15 days from laying	Control	0	0	86	4	0	0	0	90
	Experimental	0	0	82	2	3	3	0	90
Diapause eggs:									
One hour daily for 28 days	Control	1	0	88	1	3	1	0	94
	Experimental	2	2	87	0	5	0	0	96
Postdiapause eggs:									
One hour daily for 20 days		5	23	20	37	5	0	3	93
Two hours daily for 20 days		8	12	34	34	4	0	2	94
Three hours daily for 20 days		6	8	28	32	6	0	10	90
Four hours daily for 20 days		12	20	40	14	6	0	0	92
Control—2 hours daily for 20 days under glass		0	16	14	38	10	0	6	84

pieces of chorion and cuticle, indicating an impermeability to the primary radiations of ultra-violet light.

The fact that only the chorion is present for the first 6 days after the eggs are laid and that it may be removed after the fifteenth day, leaving only the cuticle, provides an excellent opportunity to study the penetration of the secondary radiations as shown by effects on the eggs. The results of irradiation with the chorion alone and with both the chorion and cuticle are presented in Table 1. The upper part of the table represents a period during the first 6 days, when the chorion alone is present. In the remainder of the table both membranes are in place. It is apparent from this table that there is no effect of the irradiation on the intact egg. In the case of postdiapause eggs that were irradiated as much as 4 hours daily for 20 days, there is still no effect.

The oxygen consumption of prediapause and postdiapause eggs after irradiation is presented graphically in Figure 1. From inspection of this figure it may be noted that the respiration of the experimental and control groups is very similar, there being no obvious effect of the ultra-violet irradiation. In view of these results the chorion has been removed from all of the eggs used in the following experiments.

2. *The effect of single exposures on dechorionated postdiapause eggs.*—Figure 2 is a graphic presentation of the changes in oxygen consumption of groups of dechorionated

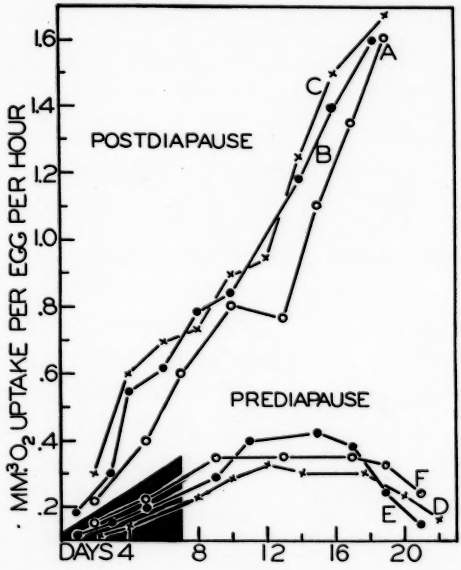

FIG. 1.—Effect of ultra-violet irradiation of prediapause and postdiapause eggs with chorion, and with chorion and cuticle present, on their oxygen consumption. In the postdiapause group, curve *A* (control) was irradiated 2 hours daily under glass, curve *B* was irradiated 2 hours daily, and curve *C* 4 hours daily. The eggs hatched at the end of this time. In the prediapause group, curve *F* is the control (2 hours daily under glass), curve *E* was irradiated 2 hours daily, and curve *D* 4 hours daily. The solid black area indicates the time during which only the chorion is present. The decreased respiration about the twentieth day is due to the normal occurrence of diapause.

eggs irradiated by single exposures of different lengths on the first day of postdiapause development. It should be noted that single exposures of less than 15 minutes produce no apparent effect. A 15-minute exposure causes a small but consistent stimulation, while there seems to be a very definite threshold between a 15- and 30-minute exposure, the latter being decidedly detrimental to the eggs. Partial recovery is evidenced after exposures of 30 and 60 minutes, but longer periods of irradiation permit practically no development. The hatching record of these eggs is shown in Table 2. It may be seen that this is in agreement with the altered oxygen consumption and shows the maximum effect in 90-minute exposures. The curious retardation in hatching of eggs irradiated 30 minutes or longer reappears more strikingly in a later experiment.

3. *The effect of daily irradiation of postdiapause eggs.*—From an inspection of Figure 3 it may be noted that postdiapause eggs are particularly susceptible to short, daily ex-

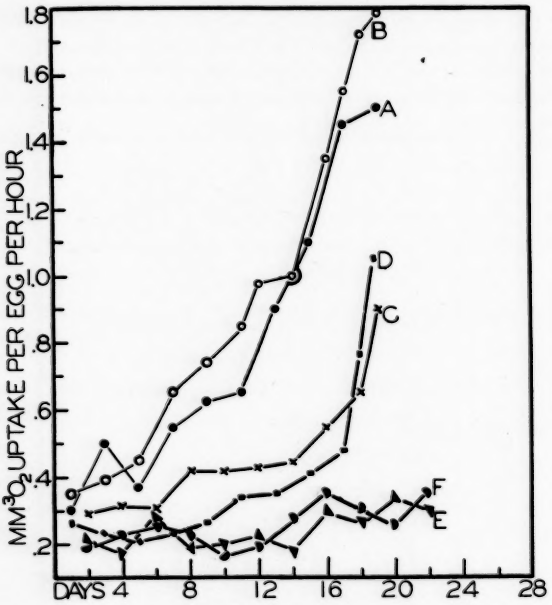

FIG. 2.—Effect of single ultra-violet irradiations on the respiration of dechorionated postdiapause eggs. The eggs were irradiated on the first day out of diapause, as follows: curve *A* (control) 2 hours under glass, curve *B* 15 minutes, curve *C* 30 minutes, curve *D* 60 minutes, curve *E* 90 minutes, and curve *F* 120 minutes.

TABLE 2

EFFECTS OF SINGLE ULTRA-VIOLET RADIATIONS OF THE AMOUNTS INDICATED ON THE FIRST DAY AFTER DIAPAUSE ON THE HATCHING OF DECHORIONATED EGGS

Values are given in percentage of eggs started and the days after diapause on which the eggs hatch are indicated.

TIME OF RADIATION	DAYS TO HATCH															
	21	22	23	24	25	26	28	30	31	32	34	37	43	47	50	Total
Control	32	20	22	4	....	....	....	....	....	....	....	....	....	....	....	78
15 minutes	22	30	14	10	4	....	....	....	....	....	....	....	....	....	....	80
30 minutes	....	8	18	6	2	2	6	....	....	4	4	....	2	....	....	52
60 minutes	....	....	....	6	....	6	6	6	6	4	4	....	....	....	....	38
90 minutes	....	....	2	2	....	....	2	2	8	....	2	4	....	....	2	24
120 minutes	....	....	2	6	....	2	4	2	6	....	....	....	4	....	4	30

posures to ultra-violet light Daily exposures of 5 seconds seem to have no effect. Fifteen-second exposures produce a slight stimulation in oxygen consumption but with a reduced hatch. Daily irradiations of 1 minute or longer greatly reduce all activity of the eggs, and only a few hatch.

4. *Recovery and increasing resistance with age.*—To study the recovery after various amounts of irradiation, ten groups of eggs were selected, and nine of these were given daily irradiations of 15 minutes for from 1 to 9 days, beginning on the first day of postdiapause development. Figure 4 is a graphic presentation of the changes in the oxygen consumption in these groups. After one or two exposures a slight stimulation is suggested. Three, four, and five 15-minute exposures permit development to about 50 per cent of normal. Six or more exposures, totaling 90 minutes or more, gradually retard development, and finally the respiration begins to decrease. Less than 5 per cent of the eggs hatch. The hatching record of this series is summarized in Table 3. Again,

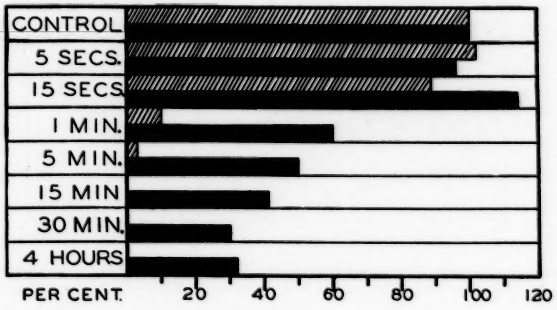

FIG. 3.—Effect of daily irradiations of amounts indicated for 18 days on the hatch and respiration of dechorionated postdiapause eggs. The diagonal-striped columns represent the percentage of hatch, and the solid columns represent the percentage of oxygen consumption, based on the controls as 100 per cent.

the group showing a slight stimulation in respiration shows also a marked decrease in hatch as compared to the control group. An even more decided retardation in hatching is demonstrated here than in Table 2.

The resistance of the eggs to ultra-violet irradiation seems to depend on their physiological condition rather than simply to increase with age. Figure 5 summarizes the results with eggs that were given single 30-minute exposures at different stages of postdiapause development and indicates at first a moderate resistance to irradiation which decreases markedly by the sixth day and from that time increases with age to a maximum about the eleventh day. The hatching record was in close agreement with these changes in oxygen consumption.

5. *The differential effect on the eggs.*—It should be considered whether the effect is the same on the entire egg or whether it is limited to the yolk or to the embryo. In this series of experiments the eggs were partially dechorionated so that only the yolk was exposed. However, during yolk engulfment the embryo would become exposed about the tenth day of postdiapause development. The results are presented graphically in Figure 6. Two groups of eggs were prepared as above described and were irradiated 1 hour daily from the first day of postdiapause. Just before the embryos became exposed,

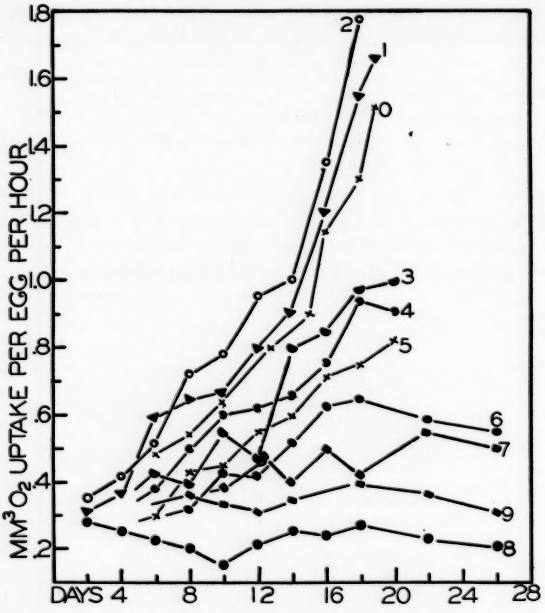

FIG. 4.—Effect on the respiration of dechorionated postdiapause eggs from one to nine daily irradiations of 15 minutes. The number of the curve indicates how many 15-minute irradiations a group has had over an equal number of days, i.e., curve *0* is the control group, curve *1* was irradiated 15 minutes on the first day out of diapause, curve *2* was irradiated 15 minutes daily on the first 2 days out of diapause, etc.

TABLE 3

EFFECT OF 15-MINUTE, DAILY ULTRA-VIOLET RADIATIONS FOR 1 TO 9 DAYS AFTER DIAPAUSE ON THE HATCHING OF DECHORIONATED EGGS

The values are given in percentage of eggs started and the days after diapause on which the eggs hatch are indicated.

No. of 15-Minute Radiations	Days to Hatch																
	17	18	19	20	21	22	24	26	28	31	32	33	37	40	43	65	Total
0	....	30	18	26	10	6	....	....	....	....	....	....	....	....	....	....	90
1	2	22	10	14	10	4	18	....	....	....	....	....	....	....	....	....	80
2	2	8	8	20	10	2	2	4	....	....	2	....	....	....	....	....	58
3	....	....	....	16	18	....	14	....	6	....	....	....	....	....	....	....	54
4	....	....	....	2	14	1	16	....	....	....	....	....	....	....	....	....	33
5	....	....	....	....	2	2	16	....	10	2	2	....	....	....	....	....	34
6	....	....	....	....	....	....	6	2	....	2	2	4	....	....	....	....	16
7	....	....	....	....	....	....	....	....	4	....	....	....	....	....	....	4	8
8	....	....	....	....	....	....	....	....	....	....	....	....	....	2	2	....	4
9	....	....	....	....	....	....	....	....	....	....	....	....	2	2	....	....	4

the irradiation of one group was continued under glass (controls), while the other group was exposed under quartz, as before. Apparently there is no effect on the respiration

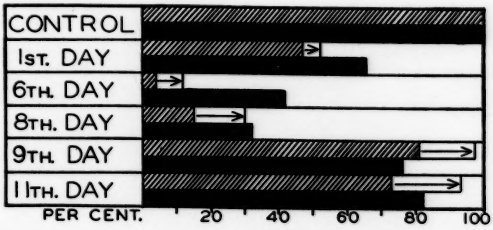

FIG. 5.—Effect of single exposures of ultra-violet light on the different ages of postdiapause eggs. The eggs were irradiated for 30 minutes on the day of postdiapause development, as indicated. The diagonal-striped columns, plus the portion occupied by the arrows, show the percentage of hatch. The solid black columns show the percentage of respiration over the entire postdiapause period. The portions occupied by the arrows show the percentage that died immediately after hatching. Percentages are calculated on the basis of the controls as 100 per cent.

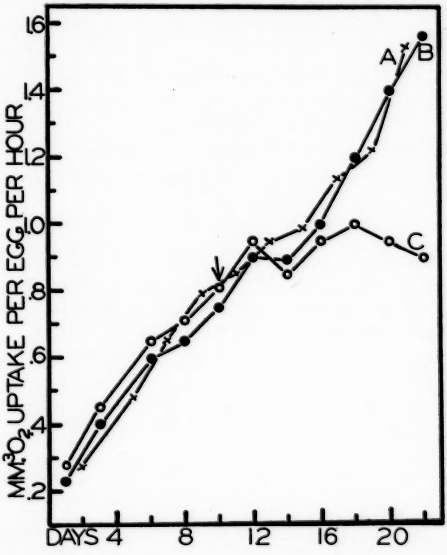

FIG. 6.—Effect of daily irradiations of 1 hour on the yolk of postdiapause eggs. Curve *A* (controls) was irradiated under glass. Curve *B* was irradiated with the yolk exposed until the tenth day(indicated by the arrow), after which it was also irradiated under glass. Curve *C* was irradiated during the entire period, with the embryo becoming exposed about the twelfth day.

until the embryo itself is exposed to the ultra-violet light, at which point there is a retardation and finally a decrease. Eggs in which only the yolk was exposed continued a normal respiration and hatched satisfactorily in contrast to irradiated embryos.

6. *The effect of ultra-violet light on the respiratory mechanism.*—Effects thus far presented have been obtained with developing eggs. It seems possible that the inhibited respiration may have been produced through effects on growth and metabolism rather than on the respiratory mechanism itself. Diapause (inactive) eggs and developing ones whose activity was temporarily and reversibly inhibited by keeping them at 5° C. were thought to offer an excellent opportunity to determine whether the ultra-violet light affected the respiratory mechanism primarily or through effects on growth, tissue structure, etc. The oxygen consumption of such eggs after irradiation is presented in Figure 7. It should be noted that in both types of eggs there was no apparent effect on the respiration while they remained inactive. None of the irradiated eggs hatched. Those that were kept at 5° C. showed a slight response when returned to developmental temperatures, and then degenerated. Such behavior tends to indicate that the effect of the ultra-violet light on respiration is produced through some intermediate factor rather

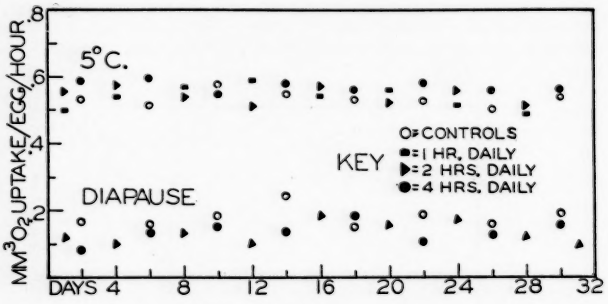

FIG. 7.—Effect of ultra-violet irradiation on the respiration of normally and experimentally blocked eggs. Four groups of eggs blocked experimentally by keeping at 5° C. are represented as follows: controls, irradiated 2 hours daily under glass; and three other groups, irradiated 1, 2, and 4 hours daily, respectively. These were 6-day postdiapause eggs. Three groups of normally blocked (diapause) eggs are shown: controls, irradiated 2 hours daily under glass, and two experimental groups, irradiated 2 and 4 hours daily, respectively. The groups are indicated in the key.

than primarily on the respiratory mechanism itself. These results are essentially in agreement with those of Boell, Ray, and Bodine (1937), indicating that effects of X-radiation on the respiration of *M. differentialis* reported by Evans (1934) were perhaps produced in this indirect manner.

DISCUSSION

In view of the fact that the ultra-violet light does not penetrate the cuticle directly, it is to be assumed that any effects were produced by secondary radiations given off by the cuticle and that these radiations were of wave-lengths longer than those applied directly to the eggs, as is usually true when radiations are absorbed and given off. Presumably, then, the effective radiations were from the region of the long ultra-violet. This region has been found to be particularly active physiologically (Duggar, 1936).

Stimulated oxygen consumption was observed in several instances (Figs. 2, 3, and 4), but in each case it was accompanied by a reduced hatch. This is thought to demonstrate that the stimulation itself was due to some physiological disruption rather than simply

to an increase in rate of the normal metabolic functions, and hence is correlated with a detrimental effect, appearing as lowered hatching percentages. Heilbrunn (1928), in studying the effects of ultra-violet light on *Amoeba*, observed a "clotting" of the protoplasm in connection with stimulation. Aside from those short exposures that produced an increased oxygen consumption, the inhibition of respiration and the decreased percentage hatch is proportional to the length of the exposure up to exposures of 2 hours, at which point a maximum effect is produced. It is further to be noted (cf. Figs. 2 and 3) that short, frequent irradiations are more effective than the same total exposure in a single irradiation. That the stimulation and inhibition occur simultaneously or are closely associated is indicated (Fig. 3) by the fact that increasing the irradiation as little as 45 seconds daily changes the results from the former to the latter.

It is generally considered that embryonic tissues become less susceptible to the action of radiation with increasing age, but various irregularities have been observed. Hinrichs (1925) found that the eggs of *Fundulus heteroclitus* were differentially susceptible to ultra-violet irradiation even in the first few minutes after fertilization, and that the regions of highest metabolic activity were most affected. Bachem and Dushkin (1935) report that the susceptibility of bacteria to ultra-violet irradiation is extremely dependent on their physiological condition. Similar results have been found with the grasshopper embryo in a study of its susceptibility at different ages (Fig. 5). Apparently there is a marked decrease in resistance to irradiation on the sixth day of postdiapause development. This period includes the completion of blastokinesis and the beginning of yolk engulfment, involving considerable activity on the part of the embryo. It has also been observed that the great majority of the embryos that are irradiated before this time and never hatch have ceased development at this point. It seems to be a period of extreme susceptibility. First-day eggs are only slightly more affected than 9-day ones. From the sixth day the resistance increases with age to a maximum about the fifteenth day.

A notable retardation in hatching has been demonstrated as an effect of ultra-violet irradiation of grasshopper eggs (Tables 2 and 3). Extreme variation in individual susceptibility is also indicated. It is a question of some interest why, if those eggs are going to hatch, some of them should hatch from 30 to 45 days later than the control groups. Similar effects, but to a lesser degree, have been noted by Nebel, Harvey, and Hollaender (1937), who found a marked inhibition in cleavage after irradiation of *Arbacia* eggs with the long ultra-violet. Unfertilized eggs could be activated to cleavage, which was also retarded.

In view of the fact, demonstrated by Bodine and Boell (1936), that the marked increase in oxygen consumption immediately following diapause occurs primarily in the yolk cells and extra embryonic material and that the yolk components constitute about 30 per cent of the total egg respiration at this time, the complete resistance of these components to ultra-violet irradiation (Fig. 6) is of considerable interest. Comparatively heavy daily exposures of yolk to ultra-violet light for 10 days, including this period of increased activity, show no apparent effect. This resistance is even more interesting when the presence of tyrosinase is considered. Bodine and Boell (1935) have shown that comparatively large amounts of tyrosinase are present in the grasshopper eggs and that practically all of it is in the yolk and its components. Narayanamurti and Ayyar (1929) report the activation of tyrosinase by ultra-violet irradiation; and Hin-

richs (1925) believes that tyrosine acts as an optical sensitizer, rendering cells susceptible to the toxic action of ultra-violet light. Keeping these considerations in mind, it seems remarkable that the yolk and its components show no effects of irradiation.

SUMMARY AND CONCLUSIONS

Neither the chorion nor the cuticle around the egg of the grasshopper, *M. differentialis*, are permeable to direct ultra-violet radiations. The cuticle seems to permit the passage of effective secondary irradiations.

Short irradiations with ultra-violet light produced increased respiration and decreased hatch. Longer irradiations inhibit both respiration and hatching in amounts proportional to the radiation. Below the maximum effect a given amount of ultra-violet radiation is more effective in short frequent exposures than in a single one.

Variations in susceptibility to ultra-violet irradiation are correlated with physiological activity rather than with the chronological age of the embryo. Periods of increased activity are most susceptible.

An extreme variation in individual sensitivity and a marked retardation in hatching have been demonstrated.

The action of ultra-violet light has been shown to be on the embryo rather than on the yolk or its components.

Altered respiration is apparently produced through changes in growth and metabolism and not by action of the radiation directly on the respiratory mechanism.

LITERATURE CITED

BACHEM, A., and DUSKHIN, M. A. 1935. A study of bacterial sensitivity to ultraviolet radiation. Biol. Bull., **69**:109–25.

BLACK, W. A. 1936. Experimental modifications of the effect of ultraviolet on protoplasm. I. *Ameba proteus*. Univ. Calif. Pub. Zoöl., **41**:75–100.

BODINE, J. H. 1929. Factors influencing the rate of respiratory metabolism of a developing egg. (Orthoptera). Physiol. Zoöl., **2**:459–82.

———. 1932. Hibernation and diapause in certain Orthoptera. II. Response to temperature during hibernation and diapause. *Ibid.*, **5**:538–48.

BODINE, J. H., and BOELL, E. J. 1934. Respiratory mechanisms of normally developing and blocked embryonic cells (Orthoptera). Jour. Cell. and Comp. Physiol., **5**:97–113.

———. 1935. Enzymes in ontogenesis (Orthoptera). *Ibid.*, **6**:263–75.

———. 1936. Respiration of embryo versus egg. *Ibid.*, **8**:357–65.

BOELL, E. J.; RAY, O. M.; and BODINE, J. H. 1937. Effect of X-rays on the oxygen consumption of embryonic cells. Radiology, **29**:533–40.

BURGE, W. E.; FISCHER, W. R.; and NEILL, A. J. 1916. The destruction of hormones, proenzymes and enzymes by ultraviolet radiation. Amer. Jour. Physiol., **40**:426–32.

CLARK, J. H. 1922. The physiological action of light. Physiol. Rev. **2**:277–309.

CROWE, M. O'L. 1936. The ultraviolet absorption spectrum curve of phthiocol, a pigment of the human tubercle bacillus. Jour. Biol. Chem., **115**:479–84.

DUGGAR, B. M. 1936. Biological effects of radiation. New York: McGraw-Hill.

ELLIS, CARLTON, and WELLS, A. A. 1925. The chemical action of ultraviolet rays. New York: Chem. Catalogue Co.

EVANS, T. C. 1934. Respiration and visible development of individual grasshopper eggs following X-irradiation. Physiol. Zoöl., **7**:556–71.

HARRIS, D. H. 1926. Observations on the velocity of the photo-oxidation of proteins and amino-acids. Biochem. Jour., **20**:288–92.

HEILBRUNN, L. V. 1928. The colloid chemistry of protoplasm. Berlin: Gebruder Borntraeger.

———. 1932. Action of ultraviolet light on amoeba protoplasm. Collecting Net, **7**:57–59.

HEILBRUNN, L. V., and DAUGHERTY, KATHRYN. 1933. The action of ultraviolet rays on amoeba protoplasm. Protoplasma, **18**:596–619.

HINRICHS, MARIE A. 1925. Modification of development on the basis of differential susceptibility to radiation. I. *Fundulus heteroclitus* and ultraviolet radiation. Jour. Morph. and Physiol., **41**:239–63.

HOLLAENDER, A., and DUGGAR, B. M. 1926. Irradiation of plant viruses and of microörganisms with monochromatic light. III. Resistance of the virus of typical tobacco mosaic and *Escherichia coli* to radiation from λ 3000 to λ 2250 A. Proc. Nat. Acad. Sci., **22**:19–24.

LAURENS, HENRY. 1928. The physiological effects of radiation. Physiol. Rev., **8**:1–91.

LILLIE, R. S., and BASKERVILL, MARGARET L. 1922. The action of ultraviolet rays on starfish eggs. Amer. Jour. Physiol., **61**:57–71.

NARAYANAMURTI, D., and AYYAR, C. V. R. 1929. Studies in enzyme action. IV. Tyrosinase I. Jour. Indian Inst. Sci., **12**:109–29.

NEBEL, B. R.; HARVEY, E. B.; and HOLLAENDER, A. 1937. The cytology of *Arbacia punctulata* activated by monochromatic ultraviolet radiation. Biol. Bull., **73**:365–66.

SLIFER, E. H. 1932*a*. Insect development. III. Blastokinesis in the living grasshopper egg. Biol. Zentralbl., **52**:223–29.

———. 1932*b*. Insect development. IV. External morphology of grasshopper embryos of known age and with known age and known temperature history. Jour. Morph., **53**:1–21.

SPIEGEL-ADOLF, M., and OSHIMA, Z. 1929. Physikalisch-chemische Untersuchungen an bestrahlten Proteinen. VI. Mitteilung weiter Studien zum spektroskopischen und biologischen Nachweis von lichtner Änderungen an Proteinen. Biochem. Zeitschr., **208**:32–44.

WYCKOFF, RALPH W. G. 1931. The killing of colon bacilli by ultraviolet light. Jour. Gen. Physiol., **15**:351–61.

THE RATE OF OXYGEN CONSUMPTION OF FROGS DURING EMBRYONIC DEVELOPMENT AND GROWTH

(Six figures)

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THE rate of oxygen consumption during development is an important factor in any analysis of the unknowns of metabolism responsible for development. For this reason many workers have attacked this problem on different species. Warburg (1908), with his work on the sea-urchin egg, was one of the first to achieve results that may be considered to approach quantitive accuracy. In 1915, with his well-known respirometer, he showed that during the development of the sea-urchin egg the respiratory rate (measured in cubic millimeters of oxygen consumed per hour) rose continuously up to gastrulation, which was as far as these observations went.

Bataillon (1896) might be considered the pioneer in this field on amphibian development. He measured carbon dioxide given off rather than oxygen consumed. Bataillon's data indicated the presence of peaks and troughs in the rate of respiration at various stages of development. Godlewski (1900) was the first actually to determine the rate of oxygen consumption of amphibian eggs. His data indicated a continuous increase in the rate. These earlier workers were handicapped by the lack of sufficiently accurate methods. Thus, Godlewski could only measure the rate of increase per day. The important matter from a physicochemical analysis of development is the instantaneous rate of oxygen consumption. An approach to the determination of this was made by Bialaszewicz and Bledowski (1915), who used the Winterstein microrespirometer on eggs of *Rana temporaria (fusca)*. With this apparatus the oxygen uptake can be determined for intervals as low as 15 minutes. By repeated observations an analysis of the instantaneous rate may be made. They concluded that there is a gradual rise in rate of oxygen consumption from fertilization until 150 hours later (when the external gills had reached their greatest development, and when a plateau had been reached in the curve relating rate of oxygen intake to time). Their data suffered, however, from observations which were too widely spaced. In 1921 Parnas and Krasinska attacked the problem anew by means of the Barcroft-Warburg respirometer. Their material included *R. temporaria (fusca)*, *R. esculenta*, and *Bufo vulgaris*. Instead of a gradual increase in rate, these workers found that the rate of oxygen consumption increased abruptly at the onset of three critical stages: gastrulation, neurulation, and appearance of external gills. Between the beginning of these stages the rate seemed to remain constant. Owing to the temperature (15° C.) at which these investigators worked, and also to the small quantity of eggs used, they determined the total oxygen consumed over a lengthy period, and calculated the rate indirectly.

Such was the conflicting status of the question when the work on which this paper is based was begun in 1934. At the time, no data were present for American amphibians. It was considered important to reinvestigate this problem as a part of a general attack on the various factors in the rate of development, especially as affected by temperature.

Since the beginning of the work, several additional papers have appeared which are relevant to the subject: Brachet (1934) on *R. fusca*, Wills (1936) on *R. pipiens*, and Stefanelli (1937) on *R. fusca*. None of these is directly concerned with the question of change of instantaneous rate with time at constant temperature. The observations of the first two are too widely spaced to determine the answer to the question of the existence of critical periods. The last paper only considers fluctuations in rate during early cleavage.

It is hoped that the data presented by this paper will aid in the elucidation of this problem. At the same time, an attempt has been made to relate the changes in rate of oxygen consumption to other variables during development, including length and weight. Studies on the growth of the amphibian embryo have been made since Davenport (1897) and Schaper (1902). Until now, however, no attempt has been made to relate embryonic growth and rate of respiration. Although no causative interrelation need be assumed, still it is important to know how these phenomena vary simultaneously. Such data should be helpful in answering the question whether the increase in the respiratory rate is due to an increase in the intensity of respiration of the separate cells or whether it is due to an increase in the number of cells.

MATERIAL AND METHODS

The eggs of *R. pipiens* were obtained after injection of anterior pituitary gland by the method described by Rugh (1937). They were fertilized artificially and kept in finger bowls until used in the experiments. In the earlier experiments the material used was timed according to stage from the data given by Atlas (1935). In the later experiments each batch of eggs was kept in an incubator at 20° C. ± 0°.5. Care was taken to avoid crowding, and the average density was not greater than 50 eggs for 200 c.c. of water (pre-gastrula stages) and 25 eggs for 200 cc. (later stages).

In the earlier experiments Great Bear spring water was used as the medium for the eggs and embryos. In later experiments an artificial pond water was made by diluting Ringer's solution with nine volumes of water. Results with this one-tenth Ringer's solution did not give any appreciable differences from that with the natural spring water.

Two types of apparatus were employed in obtaining the measurements of oxygen consumption. In the earlier experiments the Barcroft-Warburg manometric respirometer was employed. A recent outline of the theory of this apparatus is available in Dixon (1934). Two sizes of vessels were employed: a small one (volume, 8–10 cc.), which produced a change of 1 mm. in the height of Brodie's fluid for every 0.65–0.75 cu. mm. of gas consumed; and a larger one (volume, 24.5–27.0 cc.), which produced a change of 1 mm. in the height of fluid of the manometer for every 1.97–2.1 cu. mm. of gas consumed. A large number of observations was obtained with the Fenn-Winterstein volumetric respirometer, as described by Fenn (1927). The blank vessels of this apparatus were approximately 15 cc. in volume. Each millimeter excursion of kerosene drop corresponded to an uptake of approximately 0.4 cu. mm. of oxygen.

The number of eggs used in each flask varied for different stages studied. For stages up to gastrulation, 50 eggs were employed. In later stages fewer embryos were needed, and at the time of operculum formation 5 tadpoles were sufficient. It was difficult to remove the embryos from the surrounding membranes before the closure of the neural folds. It was found expedient, before this stage, to remove as much of the jelly as was

possible, by placing the eggs on a wet towel or on filter paper and cutting into the innermost layer of jelly with scissors. This leaves a small amount of jelly adhering to the vitelline membrane which, however, does not appear to affect the oxygen uptake.

Attempts were made to remove the jelly by means of 0.7 per cent KCN. This method was first described by Bataillon (1912) and was used with success on *R. fusca* by Brachet (1934). If *R. pipiens* eggs are placed in this solution at the optimum period (which is about 30 minutes after fertilization), much of the jelly is dissolved within 45 minutes; but only perhaps 10 per cent of the eggs will continue to develop normally in favorable batches, even after prolonged washing (up to 2 hours). Such normally developing eggs, however, do not differ in their oxygen consumption from those which retain small amounts of jelly which have not been exposed to KCN. This confirms Brachet (1934). The difference in behavior to KCN is probably a species difference.

The procedure finally adopted in preparing the experiment was the following: Jelly was removed when present. Observations were then made regarding stage and size. The latter was determined, for the early stages (up to 5 mm.), as the greatest diameter or length, by means of an ocular micrometer in a compound microscope. For lengths greater than 5 mm. a millimeter ruler was used under a dissecting binocular. The eggs or embryos were then placed in a 10 cc. graduated cylinder, in which they were washed with several volumes of one-tenth Ringer's solution. All excess water was then drawn off, and 2 cc. of clean one-tenth Ringer's solution were added. The volume of eggs plus water was thus determined. Two-tenths of a cubic centimeter of 5 per cent KOH was now added to the side arm of the Warburg or Fenn flask. The eggs and water were then pipetted into the main chamber of the flask. This was now connected with the tap of the manometer or volumeter and placed in the constant-temperature bath. The apparatus was shaken with stopcocks open to the air for 15 minutes, to attain temperature equilibrium. The stopcocks were then closed and the shaking continued. The rate of shaking in most experiments was eighty-five complete oscillations per minute. Until closure of the neural folds the rate of shaking may be decreased to sixty per minute without affecting the rate of uptake. The amplitude of shaking in the Warburg manometer experiments was approximately 9.5 cm.; in the Fenn apparatus it was about 5 cm. The temperature of the water bath was kept constant at 19°.8 C. ± 0.01°. One series of experiments was carried out at 14°.8 ± 0.02°.

In several series of eggs, along with the rate of oxygen consumption, the wet and dry weights were determined either of the eggs used in the experiment or of eggs from the same batch and at the same stage of development. Wet weight was determined by weighing the eggs or larvae (minus their membranes) on a watch glass or slide, after as much water as possible has been absorbed from the vicinity of the egg by means of filter paper. Great care must be taken to prevent additional loss of water by evaporation. The eggs were then placed in an oven at 110°–120° C. and left overnight. The dry weight was then determined.

RESULTS

Eggs which were removed from the uterus and placed in the respirometers within 40 minutes after fertilization began to consume oxygen immediately. There was no sign of the positive pressure reported by Bialaszewicz and Bledowski, which was attributed by these authors to the evolution of an oxygen-nitrogen gas mixture, resulting from the difference between the pressure of these gases in the body and in the atmosphere.

No detailed comparison of the respiration of unfertilized and fertilized eggs was made, since this question has been fully investigated by previous workers, who are all agreed on the occurrence of a slight increase in rate after fertilization. As the result of experiments on batches of eggs from three different females, it was found that the average oxygen consumption of 100 unfertilized eggs per hour was 5.86 cu. mm., while that of fertilized eggs from the same females was 5.98 cu. mm.

TABLE 1

RATE OF OXYGEN CONSUMPTION STARTING 2.5 HOURS AFTER FERTILIZATION

Time of Observation	Calc. Rate for 1 Hr. per 100 Eggs (Cu. Mm.)	Time of Observation	Calc. Rate for 1 Hr. per 100 Eggs (Cu. Mm.)
4:30–5:30	4.98	6:00–7:00	5.22
4:45–5:45	5.38	7:00–8:00	6.12
5:00–6:00	5.44	8:00–9:00	5.74
5:15–6:15	5.46	8:30–9:30	5.82
5:30–6:30	5.92		

During cleavage the rate of respiration tends to rise, as can be seen from Table 1 and as has been indicated previously by Brachet (1934). This table shows the observed rates of oxygen consumption, with the Fenn volumeter, per 100 eggs for one batch on which observations started at the beginning of first cleavage. Whether there are fluctuations corresponding to the cleavage rhythm, which have been reported by Brachet (1934) and Stefanelli (1937), could not be determined in the present instance, since the

TABLE 2

RATE OF OXYGEN CONSUMPTION DURING GASTRATION

Time of Observation	Calc. Rate for 1 Hr. per 100 Eggs (Cu. Mm.)	Time of Observation	Calc. Rate for 1 Hr. per 100 Eggs (Cu. Mm.)
3:00–4:00	11.70	4:02–5:02	12.02
3:17–4:17	11.70	4:17–5:17	12.10
3:30–4:30	11.62	4:32–5:32	12:42
3:45–4:45 (beginning of gastration)	11.94		

apparatus was not sufficiently sensitive to note such fluctuations. The fluctuations which are seen to occur in Table 1 were noted in later stages and are assumed to be due to experimental errors. Despite the fluctuations the rate of respiration continues to rise, until, before gastrulation, it is double the rate during early cleavage. In Table 2 are shown the observations on the same eggs, started before the beginning of gastrulation and continued until some time after gastrulation had begun. It will be seen from these data that there is no prolonged period over which the rate of oxygen consumption

remains constant, as Parnas and Krasinska (1921) concluded. Nor does the rate jump abruptly at the beginning of gastrulation, but tends to mount steadily.

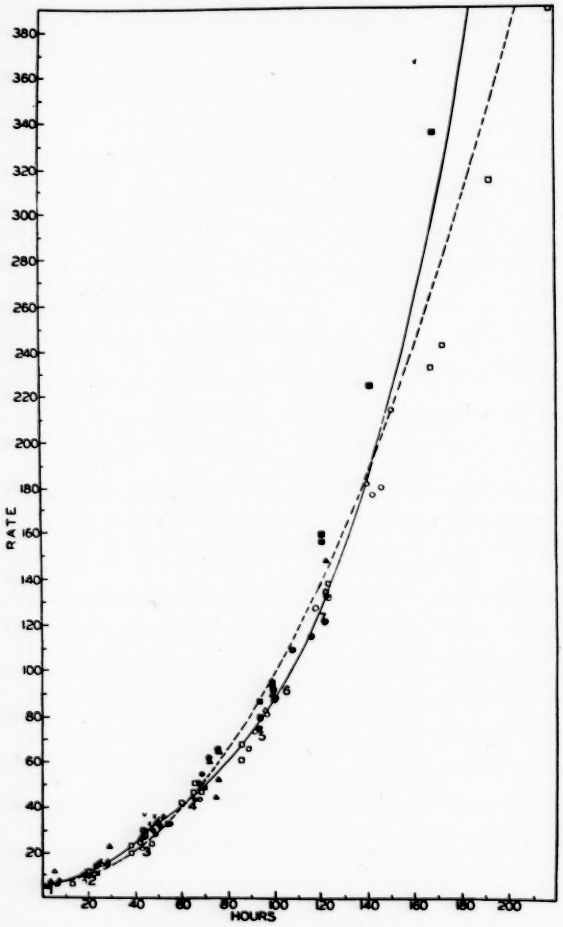

FIG. 1.—Rate of oxygen consumption per 100 eggs per hour during development. The abscissas represent hours after fertilization. The broken curve is for the equation $y = 0.00925t^3$. The full curve from 0 to 47 hours is for the equation $y = 5.31e^{0.038t}$. The full curve after 47 hours has the equation $y = 31e^{0.019(t-47)}$.

A detailed study of the combined data indicates a similar conclusion for the later stages. In Figure 1 are presented the results obtained with the Fenn volumeter. The abscissas indicate hours of development; and the ordinates, rate of oxygen consumption for 100 eggs per hour. Similar symbols are for eggs from the same female. The numerals

near the points refer to the following stages of development: (1) first cleavage; (2) beginning of gastrulation; (3) slit blastopore to neural plate; (4) neural folds fusing; (5) hatching (about 4.5 mm. length); (6) heart beat visible (about 5 mm.); and (7) gill branches forming, circulation visible (about 6 mm.). The data obtained with the Warburg apparatus show similar trend but are, on the whole, more variable, and have been omitted in order to prevent confusion. Drawn through the data are two curves. The broken single curve is for the relation $y - a = kt^2$, which was found by Bialaszewicz and Bledowski (1915) to approximate their data (y = rate of oxygen consumption; a = rate of unfertilized eggs; k = constant determined by the data; and t = time). This curve also approximates the present data but is not satisfactory, particularly in the region from 40 to 55 hours, when most of the observed data are above the curve, and in the region from 95 to 120 hours, when most of the observed data are beneath the curve.

The full curve consists of two components. This curve (up to 47 hours) is drawn in an attempt to fit the data with the exponential equation $y = Ae^{kt}$, in which y = rate of oxygen consumption of 100 eggs per hour; A = rate of oxygen consumption at fertilization (a theoretical value obtained in fitting this equation, but approximately equal to the observed value); k = a constant determined by the data; and t = the time in hours after fertilization. To fit the data after 47 hours, the constants must be changed. The values of these constants is indicated in the legends. If this equation represents the correct relations between rate of respiration and time, there must be a slight discontinuity in the orderly increase of rate after the onset of neural-fold formation (after 47 hours). A more marked break was suggested by the data obtained with the Warburg apparatus and an attempt to check this was the original stimulus for the work with the Fenn apparatus.

One difference between the work of Parnas and Krasinska and that of others is the fact that the former workers made their observations at a lower temperature: they worked at 15° C. For this reason one series of data was collected as follows: The oxygen uptake was noted at a temperature of 14°.8 for 1–2 hours. The stopcocks of the manometers were then opened, and the temperature of the bath was changed to 19°.8. The manometer vessels were equilibrated for 1 hour at this temperature, and the stopcocks were then closed. Oxygen consumption was then determined at this second temperature on the same eggs. In this way it was possible to study the effect of temperature on the same material at approximately the same stage of development.

In Figure 2 the results of this parallel run are given. The curves for the 20° series are for the same exponential equations as those in Figure 1. The curves for the 15° series merely follow the data. No equation was determined for them. The dotted curve is one derived from the 15° data and represents the probable rate of increase of respiration of a batch which would develop at 15° throughout. Although the data are not sufficient to exclude the possibility of abrupt changes at critical stages, still their general course indicates the likelihood of a gradual increase in rate at this temperature also. These data further tend to corroborate the possibility of a slight discontinuity in the neighborhood of 50 hours. The temperature coefficient (Q_5) is approximately 1.8 for each stage. There is, however, insufficient data to determine whether there are any significant changes during development. A value for the Q_5 for development over this period can be obtained by interpolation in Figure 5 of the paper of Atlas (1935). This indicates a value of 1.88 for the Q_5 of development. Within the limits of experimental error these are

sufficiently close to indicate that the temperature coefficients of these simultaneous events are close together, if not identical. Although the temperature coefficients are higher than those of Tyler (1936) for various invertebrate eggs, they agree with his results in that the coefficients are practically identical for rate of development and for rate of oxygen consumption.

It was also considered desirable to obtain data from *R. sylvatica*. The eggs of this species are laid earlier, and generally at a lower temperature than those of *R. pipiens*. In this regard and in their heat tolerance they resemble *R. fusca* (both develop normally between 2° and 22°.5, whereas *R. pipiens* has a normal development range between 6° and 30°). Experiments were carried out on the eggs of *R. sylvatica*, which were collected in ponds near New York in March and April, 1937. The details of the experiments were identical with those given for *R. pipiens;* and the general conclusions are similar, in that no indications of abrupt changes appear. The data are plotted in Figure 3, in which are included the points (*B*) which represent the data for *R. fusca* (Bialaszewicz and Bledowski, 1915, Fig. 4, p. 462) and the curve for *R. pipiens* (this paper, Fig. 1) for comparative purposes. The *R. sylvatica* data approximate those for *R. fusca*. The rate for each of these species is higher than that observed for *R. pipiens* at identical hours after fertilization. The

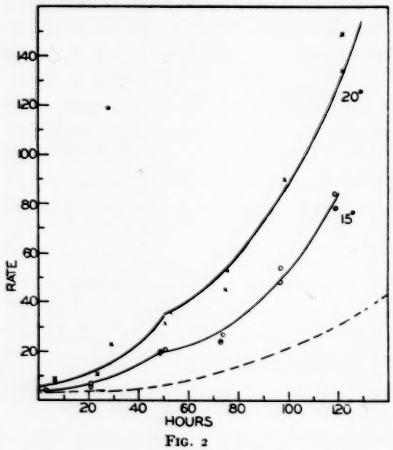

FIG. 2

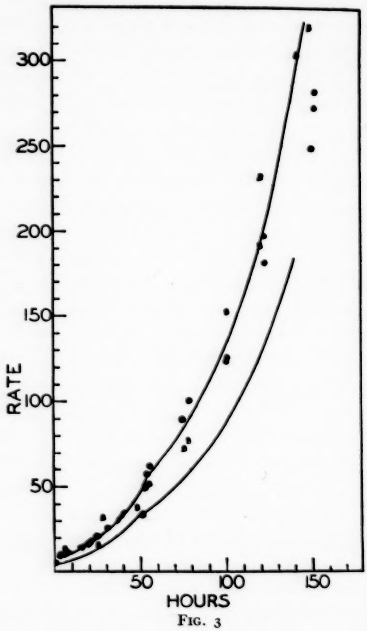

FIG. 3

FIG. 2.—Rate of oxygen consumption per 100 eggs per hour during development at 20° and 15° C. The broken curve is a calculated curve showing the rates of oxygen consumption related to time taken to develop at 15° C.

FIG. 3.—Rate of oxygen consumption per 100 eggs per hour during development of three species of frogs. The black circles are for *R. sylvatica*. The symbol *B* is for data from *R. temporaria* (*fusca*), from Bialaszewicz and Bledowski (1915). The lower curves are for *R. pipiens* from Fig. 1. The upper curves are $y = 7.5e^{0.038t}$ up to 53.3 hours and $y = 56e^{0.019(t-53.3)}$ after 53.3 hours.

dotted curves are fit to the equation $y = Ae^{kt}$, in which the terms have the same meaning as above (Fig. 1); and again there is an indication of a slight discontinuity in the neighborhood of 50 hours.

Data on the growth of these embryos are presented in Figure 4. The abscissas represent hours of development. The upper ordinates represent millimeters (longest diameter up to 50 hours, length after 50 hours); the lower ordinates represent milligrams wet weight. It will be seen that the length dimension increases slowly, and approxi-

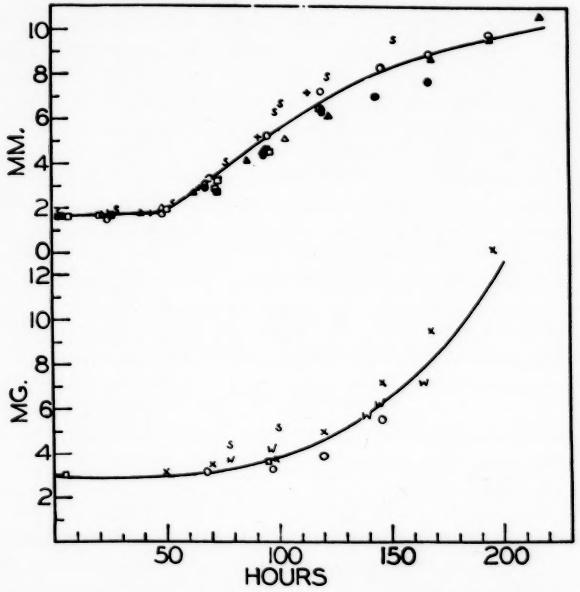

FIG. 4.—Growth of embryos and larvae during development. Similar symbols indicate data from the same batch of eggs. *S* represents data for *R. sylvatica; W* represents data for *R. sylvatica* from Williams (1900), obtained in the present form by interpolating from Fig. 6 of this paper into the upper curve of this figure. All other points are for *R. pipiens*. MM. = length in millimeters; MG. = milligrams wet weight.

mately linearly from 1.5 mm. at zero hours of development to 1.8 mm. at 50 hours (which is at the middle of the neural folds stage). Starting with this stage, the rate of increase of length becomes rapid; but at about 120 hours (when gill circulation has recently begun) the rate of growth of length decreases. The *R. sylvatica* data (indicated by the letter *S*) follow the same general course except that they are slightly in advance at each time-interval. The curve is for the smoothed data of *R. pipiens*.

In the lower curve of Figure 4 is represented the change in wet weight during development. The wet-weight data indicate that there is only a slight uptake of water before 50 hours, with a more rapid increase thereafter. Represented are the observed points from two batches of eggs. The curve drawn represents the average values obtained from many other batches of eggs. Also included are original observations (*S*) made for *R.*

sylvatica and the data (*W*) of Williams (1900); also for *R. sylvatica* (obtained by interpolation from Fig. 6, this paper). The data of Williams are of interest since they indicate that, although the initial wet weight is higher for *R. sylvatica* than for *R. pipiens*, the relation is reversed later, apparently because of the more rapid intake of water by the latter species. If we compare the two curves of this figure, it becomes apparent that increase in length precedes increase in wet weight and follows an entirely different course.

DISCUSSION

Earlier work on the development of various eggs has indicated a rise in the rate of oxygen consumption in most cases. Although there are many gaps in previous data, the evidence in general tends to indicate that there is a gradual increase in rate of respiration during development, without any abrupt changes. This is demonstrated by data for echinoderms by Warburg (1915), Meyerhof (1911), Rapkine (1927), and Ephrussi (1933); for the bryozoan *Aplysia* by Buglia (1908) and Meyerhof (1911); for insects by Fink (1925); for the chick by Bohr and Hasselbach (1900) and Murray (1925); for gastropods by Baldwin (1935); for annelid eggs by Whitaker (1933).

The earlier work on amphibian eggs also supported a continuous increase (Godlewski, 1900; Bialaszewicz and Bledowski, 1915). However, the work of Parnas and Krasinska (1921) tended to indicate periods during which the respiration remains constant, followed by abrupt changes in rate. Parnas and Krasinska concluded that the rate rose abruptly at the beginning of gastrulation, neurulation, and external gill growth, with no noticeable changes in between. Hyman (1921) has presented data for *Fundulus* which indicate a decline in rate after gastrulation.

In the present paper an attempt has been made to reinvestigate this question for amphibian eggs. As can be seen from the data in Tables 1 and 2 and Figures 1, 2, and 3, there is no indication of abrupt changes. The evidence seems to indicate a gradual rise. An examination of the data of Parnas and Krasinska leads to some clues as to the deviation of their views from those of this paper. Probably the greatest factor, indicated by their data, which contributes to their conclusions arises from the measurement of *total* oxygen uptake from the beginning of the experiment (which lasted over 4 days in some cases). As a result their data concerning rate of oxygen consumption are obtained indirectly from the total oxygen consumed. Such a procedure might be countenanced if the observations were sufficiently close to permit the drawing of an accurate curve. But there are large gaps in the data in many cases. This, together with the fact that the temperature at which the experiment was conducted showed fluctuations as much as 2° in some cases, might account for the particular conclusions that these workers reached.

The gradual increase in the rate of respiration is in harmony with observations of embryologists which indicate a continuous increase in cell numbers during the period of development that has been studied in this paper. The increase in rate of respiration of the embryo, taken as a unit, reflects the growth of cell population according to the view of the present author. The question arises whether there is any simple relation which can describe the changing rate of respiration. As indicated in Figure 1, the data which have been obtained can be described by the equation $y = 5.31e^{0.038t}$ from 0 to 47 hours of development, in which the terms have the meaning defined above. The chief value of the use of this equation is that it offers the working hypothesis that during early development the respiratory mechanism is being built up at a uniform rate, and that the

quantity of respiratory material present at any instant is greater by a constant percentage than the amount present during the immediately preceding instant.

After 47 hours there is a change in the value of the constants of the foregoing equation, as indicated in Figure 1. This change may be correlated with other changes which are occurring in the embryo at this stage, which corresponds to the onset of neural-fold formation. One of these changes is the increase in length which begins to occur about this time, as shown in Figure 4. The relation between rate of respiration and length is brought out in Figure 5. Here, on the lower abscissa, is plotted length in millimeters; and on the ordinate scale, rate of respiration. Length refers to total body length from tip of head to tip of tail; however, before the body axis is formed, length refers to the diameter of the egg. The plot shows that the rate of respiration increases rapidly, relative to length during early development. However, when length begins to increase more rapidly (at about 50 hours, or 2.1 mm. in Fig. 4) the rate of respiration follows the increase of length in accordance with the equation $y = 40e^{0.28(L-2.1)}$. Here y = rate of respiration of 100 eggs per hour, e = the base of natural logarithms, L = length in millimeters. The fit of this equation permits the hypothesis that after neural-fold formation the increase in rate of respiration becomes a constant percentage of the increase in length. Since the increase in length of these embryos is due largely to growth of the tail (Atlas, 1935, p. 304) and since the growth of the tail is due largely to an increase in cell number by cell division, the implication is that there is a constant percentage increase in cell number when the length of the embryo increases, and a resultant constant percentage increment in rate of respiration. According to this interpretation, the growth of the respiratory function is probably due mainly to an increase in total number of cells rather than to an increase in intensity of respiration of individual cells.

During the early growth of the frog larvae, one of the outstanding changes is the increase in water content, as shown in Figure 4. It is interesting to note how the rate of respiration behaves with respect to this variable. Therefore, in Figure 5 there is also plotted the rate of respiration with increase in wet weight (expressed in milligrams on the upper abscissa). It will be seen that the rate of respiration increases more rapidly relative to wet weight at first, that then the rate of increase slows up, and that during later stages the rate of respiration tends to approach an asymptote. This would tend to indicate that the rate of respiration is not increasing proportionately to increase of the volume of the embryo and that much of the water which is entering is not made part of the respiratory mechanism.[1] It must not be inferred from this, however, that the water is actually impeding the increase in the amount of respiratory mechanism. This follows from a comparison of Figures 5 and 6. In Figure 6 is plotted the relation between wet weight and length for *R. pipiens*, *R. sylvatica*, and *R. fusca*. The letters indicate the authorities from which the data were derived. It will be observed that, after 5 mm., the larvae of *R. pipiens* have more water, at equal lengths, than do those of *R. sylvatica*. In Figure 5 is plotted the rate of respiration of larvae of *R. sylvatica* at different lengths. Although the equation is slightly different for *R. sylvatica* than for *R. pipiens*, we can observe that for equal lengths of larvae the rate of respiration is approximately equal for the two species (within the limits of experimental error). In other words, despite the

[1] In Figure 6 it is shown that the change due to dry weight is negligible and may be omitted from this discussion. The change in wet weight is therefore a measure of the increase in water content.

greater amount of water for equal lengths which the larvae of *R. pipiens* have, they respire at the same rate as those of *R. sylvatica*.

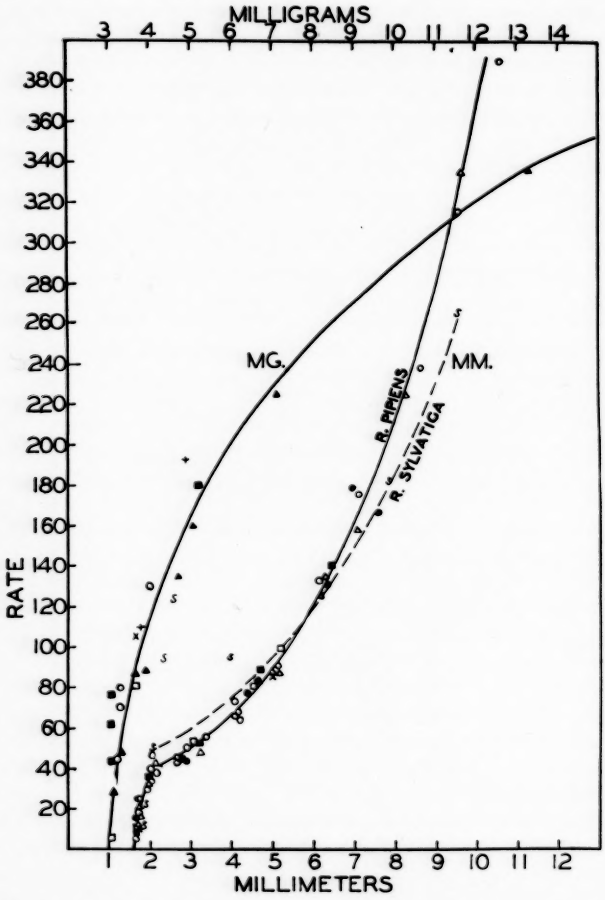

FIG. 5.—Relation between rate of oxygen consumption per egg per hour and length and wet weight. The symbol *S* and broken curve are for *R. sylvatica*. The *R. pipiens* rate-length curve is for the equation $y = 40e^{0.28(L-2.1)}$. The *R. sylvatica* curve is for $y = 50e^{0.22(L-2.1)}$.

Another matter of interest in connection with Figure 6 is that the points of *R. sylvatica* and *R. fusca* fall on approximately the same curve. This may be of significance in connection with the data presented in Figure 3 in which one can see that the rates of respiration at equal times after fertilization also correspond for these species. The two sets of data, however, cannot be further analyzed, since there are no observations at hand

which indicate the rate of length increase of *R. fusca* at a temperature of 20° C. For this reason a study of respiration at different lengths is not possible for *R. fusca*.

Also plotted in Figure 6 are the changes in dry weight which have been observed for *R. pipiens* (*x*) and for *R. sylvatica* (*w*) (data of Williams). The lines drawn connect observations made on eggs of *R. pipiens* taken from the same females. The two outer lines represent the maximum and minimum weights observed, while the middle line is from an intermediate batch of eggs. As has been noted by previous authors, the dry

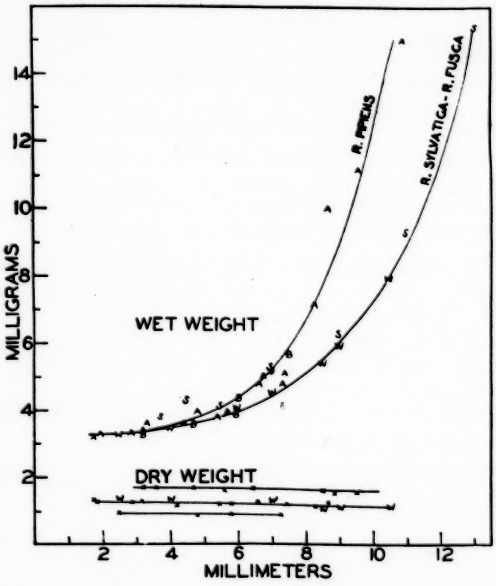

FIG. 6.—Relation of wet and dry weight to length in millimeters. Upper curve for wet weight; lower curve for dry weight. *A*, data for *R. pipiens* this paper; *B*, data for *R. temporaria* (Bialaszewicz, 1908); *S*, data for *R. fusca* (Schaper, 1902); *W*, data for *R. sylvatica* (Williams, 1900).

weight of the frog's egg diminishes during development (in the present case from a mean of 1.3 mg. at the beginning of development to one of 1.1 mg. when the external gills are covered or a difference of 0.2 mg.). It will be seen that the total loss throughout development is not appreciably larger for the eggs with more initial dry matter than for those with less. The total amount lost seems to be proportional to the length rather than to the initial dry weight.

By graphical integration of the full curves of Figure 1 the total amount of oxygen consumed during the first 200 hours of development at 20° C. can be calculated. This is approximately 250 cu. mm. for each embryo. The amount of oxygen necessary to oxidize 0.2 mg. of the dry weight of the embryo would be 149,191, or 400 cu. mm., if glucose, protein, or fat, respectively, were the primary material being oxidized (Hawk and Bergeim, 1937). From these figures it is likely that a certain amount of fat is oxi-

dized during the period studied. However, since the respiratory quotient is approximately 1 from gastrulation to hatching (Brachet, 1934), very little fat is burned during this period. The greater amount of fat must therefore be oxidized between hatching and the covering of the external gills. This is an indirect confirmation of the work of Bialaszewicz and Mincovna (1921) on *R. fusca*, who found that the disappearance of fat does not begin until after hatching of the frog larvae. This also implies that the respiratory quotient probably falls after hatching.

SUMMARY

1. The rate of oxygen consumption increases gradually, without sudden jumps, during the development of *R. pipiens* and *R. sylvatica* from fertilization to overgrowth of the operculum (Figs. 1 and 3).

2. The relation between the rate of oxygen consumption per hour per embryo and time may be expressed by the exponential equation $y = Ae^{kt}$, providing a change of constants is introduced for data after the neural-fold stage.

3. After the neural-fold stage the relation between rate of oxygen consumption (y) and length (l) of embryo may be expressed by the equation $y = Ae^{Kl}$ (Fig. 5).

4. The temperature coefficient (Q_5) is approximately the same (1.8) for rate of development and rate of oxygen consumption during development of *R. pipiens* at 15° C. and 20° C. (Fig. 2).

5. Increase in length of embryo precedes the rapid increase in water intake (Fig. 4).

6. At equal lengths *R. pipiens* and *R. sylvatica* respire at the same rate (Fig. 5) even though the *R. pipiens* larvae have more water.

7. The increase in rate of respiration per embryo is a function of the cell number rather than of the intensity of respiration of individual cells.

8. The total oxygen consumed during the first 200 hours of development of *R. pipiens* at 20° C. is approximately 250 cu. mm. The total decrease in dry weight is approximately 0.2 mg. From this it is deduced that there is probably a combustion of fat after hatching.

The author wishes to acknowledge his indebtedness to the department of zoölogy of Columbia University for facilities to carry on this work, and to Professor L. G. Barth, who helped in its progress. Some of the observations were made with the aid of Mr. N. Stevens.

LITERATURE CITED

ATLAS, M. 1935. The effect of temperature on the development of *Rana pipiens*. Physiol. Zoöl., **8**:290–310.

BALDWIN, ERNEST. 1935. The respiratory quotient of developing gastropod eggs. Jour. Exper. Biol., **12**:27–35.

BATAILLON, E. 1896. Nouvelles recherches sur les mécanismes de l'évolution. Les premiers stades du développement chez les poissons et les amphibiens. Arch. zool. expér. et générale, **5**:281–317.

———. 1912. La Parthénogénèse des amphibiens et la "fécondation chimique" de Loeb. Etude analytique. Ann. sci. nat., n.s., **16**:249–307.

BIALASZEWICZ, K. 1908. Beiträge zur Kenntnis der Wachstumsvorgänge bei Amphibionembryonen. Bull. internat. Acad. sci., Cracovie. Cl. des sci. math. et nat., pp. 783–835.

BIALASZEWICZ, K., and BLEDOWSKI, R. 1915. The influence of fertilization on the respiration of eggs. Pro. Sci. Soc. Warsaw, **8**:429–73.

BIALASZEWICZ, K., and MINCOVNA, M. 1921. Sur le métabolisme des principes gras et azotés aux premiers stades du développement de la grenouille. Trav. du Lab. de physiol. de l'Institut Nencki, 1:1–23.

BOHR, CHR., and HASSELBACH, K. 1900. Ueber die Kohlensäureproduction des Hühnerembryos. Skandinav. arch. f. physiol., 10:149–73.

BRACHET, J. 1934. 1. La Respiration et la glycolyse de la segmentation à l'éclosion (*Rana fusca*). Arch. de biol., 45:611–727.

———. 1935. 2. La Respiration de l'œuf pendant la fécondation et la mitose (*Rana fusca*). *Ibid.*, 46:1–24.

BUGLIA, G. 1908. Sullo scambio gassoso delie uova di *Aplysia limacina* nei vari periodi dello siviluppo. Arch. di fisiol. 5:455–70.

DAVENPORT, C. B. 1897. The role of water in growth. Proc. Boston Soc. Nat. Hist., 28:73–84.

DIXON, M. 1934. Manometric methods. Cambridge University Press.

EPHRUSSI, B. 1933. Contribution à l'analyse des premiers stades du développement de l'œuf. Action de la température. Arch. de biol., 44:1–147.

FENN, WALLACE O. 1927. The gas exchange of nerve during stimulation. Amer. Jour. Physiol., 80:327–46.

FINK, DAVID E. 1925. Metabolism during embryonic and metamorphic development of insects. Jour. Gen. Physiol., 7:527–43.

GODLEWSKI, E. 1900. Ueber die Einwirkung des Sauerstoffs auf Entwicklung und über den Gaswechsel in den ersten Entwicklungstadien von *Rana temporaria*. Bull. Internat. de l'Acad. Sci., Cracovie, pp. 232–55.

HAWK, P. B., and BERGEIM, OLAF. 1937. Practical physiological chemistry (11th ed.). P. Blakiston's Son & Co. Philadelphia.

HYMAN, LIBBIE H. 1921. The metabolic gradients of vertebrate embryos. I. Teleost embryos. Biol. Bull., 40:32–73.

MEYERHOF, OTTO. 1911. Untersuchungen über die Warmetönung der vitalen Oxydationsvorgänge in Eiren. Biochem. Zeitschr., 53:280–328.

MURRAY, H. A. 1925. Catabolism. Chemical changes in fertile eggs during incubation. Selection of standard conditions. Jour. Gen. Physiol., 9:1–37.

PARNAS, J. K., and KRASINSKA, S. 1921. Über den Stoffwechsel der Amphibienlarven. Biochem. Zeitschr., 116:108–37.

RAPKINE, LOUIS. 1927. Les Echanges gazeux et la nature des oxydations chez l'œuf fécondé d'oursin (*Paracentrotus lividus*). Compt. rend. Soc. biol., 97:143–49.

RUGH, R. 1937. Ovulation induced out of season. Science, 85:588–89.

SCHAPER, A. 1902. Beiträge zur Analyse des tierischen Wachstums. Eine kritische und experimentelle Studie. Arch. f. Entw'mech., 14:307–401.

STEFANELLI, ALBERTO. 1937. A new form of micro-respirometer with a note on the effect of cleavage on the respiration of the egg of *Rana*. Jour. Exper. Biol., 14:171–78.

TYLER, A. 1936. Comparison of the rates of oxygen consumption and development at different temperatures of eggs of some marine animals. Biol. Bull., 71:82–100.

WARBURG, O. 1908. Beobachtungen über die Oxydationsprozesse im Seeiglei. Zeitschr. f. physiol. Chem., 57:1–16.

———. 1915. Notizen zur Entwicklungsphysiologie des Seeigeleis. Pflügers Arch., 160:324–32.

WHITAKER, D. M. 1933. On the rate of oxygen consumption by fertilized and unfertilized eggs. *Chaetopterus* and *Arbacia punctulata*. Jour. Gen. Physiol., 16:475–95.

WILLIAMS, S. R. 1900. The specific gravity of some fresh water animals in relation to their habits, development and composition. Amer. Nat., 34:95.

WILLS, IRWIN A. 1936. The respiratory rate of developing amphibia with special reference to sex differentiation. Jour. Exper. Zoöl., 73:481–510.

LIGHT INTENSITY AND MELANOPHORE RESPONSE IN A CYPRINID FISH[1]

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THE pigmentary responses of fishes which show adjustment to background are known to be in accordance with the degree of contrast between the background and the rest of the environment under all ordinary circumstances. The intensity of illumination appears to be relatively unimportant in these responses. Such men as Sumner (1911), Mast (1914), Murisier (1921), Koller (1934), and others have concluded, in general, that the shade assumed by normal fishes is almost independent of the intensity of the light to which they are exposed. While Duspiva (1931) found marked differences in the shade of embryonic and early larval individuals of certain species, these fishes showed no clear response to background, and thus formed no exception to the general rule.

The question naturally arises whether or not the rate of color change differs at different intensities of illumination, even though the amount of change may be approximately constant. When the experiments to be described were undertaken, however, no accounts of quantitative work dealing with this point could be found in the literature. It seemed desirable, therefore, to attempt to determine the time required for melanophore change at each of several different light intensities.

The river chub, *Nocomis biguttatus* Kirtland, was chosen as the experimental animal, chiefly because of its convenient size and striking melanophore change. The individuals used, all of which were obtained from a Minneapolis bait-dealer, ranged in length from about 11 to 15 cm. When kept for any considerable period, they were occasionally fed chopped liver, earthworms, or very small minnows.

All trials were made in a cylinder consisting of a wooden frame, 40 cm. in diameter, covered with heavy, light-proof, black paper. The top of this cylinder was covered by one or more sheets of opal glass; and when still greater reduction of light intensity was necessary, sheets of pure white bond paper were inserted between the glass plates. The source of light was a Mazda lamp supported directly above the center of the cylinder by a crossarm attached to an upright pipe standard having a height of about 1½ meters above the cylinder. The crossarm could be clamped at any desired distance above the cylinder, up to the end of the pipe. In this way, exact adjustments of the light intensity within the cylinder were possible by varying the number of sheets of opal glass and paper and the distance of the lamp above them. Two clear Mazda lamps were used—1,500 watts for the highest intensity, 300 watts for all others.

The fish to be tested were placed in a white enamel or dead-black glass dish about 20 cm. in diameter and 8 cm. deep, containing 4 cm. of water. This depth of water was about the minimum in which the fish would remain reasonably quiet under the experi-

[1] I am indebted to Dr. D. E. Minnich, chairman of the department of zoölogy of the University of Minnesota, for his aid in securing materials and facilities for this investigation, as well as for criticisms both of the experimental work and the interpretation of its results.

mental conditions. The surface of the water was 25 cm. from the lower side of the diffusing opal glass. Openings made in the side of the cylinder were covered with several layers of black cloth in such a way that both hands could be introduced to manipulate the dishes containing the fish without admitting additional light.

The intensities used were 100, 10, 1, 0.1, and 0.01 foot-candles as determined by the use of a Weston photronic illuminometer at a position corresponding to the surface of the water.[2] The actual intensity reaching the fish in the water was, of course, somewhat less. Water temperatures were kept as constant as possible, but varied between 14° and 19° C. during most of the work. Part of the time a room was available in which the temperature varied only between 15° and 18°, and the fish were then kept in this room at all times.

The relatively crude method of direct observation of individual fish was used. The time was taken from the moment when the fish were placed upon the background—black or white, as the case might be—until it was judged that change was complete. Obviously, this method offers too great possibilities of error for very exact results; but because of the scarcity of material and for various other reasons, it seemed to be the only feasible method.

No standard of comparison could be devised which proved any more satisfactory than the subjective judgment of the individual fish. It was found with practice, however, that results could be obtained with a given individual under a given set of conditions which agreed surprisingly well. It is probable, to be sure, that the absolute end-point was frequently not obtained; but the remaining change which might have taken place in any particular fish was, at least, nearly negligible as far as general appearance was concerned. The results, therefore, appear accurate enough for comparative purposes.

Observations were made by quickly removing the filters covering the top of the cylinder, and at the same time changing a double-throw switch, which cut off the original light and substituted the light from another 300-watt lamp at a fixed distance. Thus, observations were always made at the same illumination. Because of the very rapid color change of this minnow, the condition of the fish then had to be judged within a few seconds as either completely or incompletely changed. Later tests were then made for a shorter or longer time, depending on whether change was complete or not, until the time limits within which the particular fish would and would not undergo complete change had been determined.

It was found necessary to put each fish through a period of preliminary "practice" lasting for a week or two before using it for the tests. As noted by Pouchet (1876) and many later investigators, the time required by fishes for color change can often be considerably reduced by such practice, and it was found that *Nocomis* in this respect formed no exception to the rule. Unless the fish were first put through this practice period, the time required for color change would continue to decrease for a considerable time. The practice period also served several other purposes. It allowed the experimenter to become familiar with the appearance of the individuals, and thus promoted more accurate judgment as to the completion of melanophore change. It made the fish much more tame and easy to handle in critical situations. Finally, it made the appearance of

[2] The lowest intensity used may not have been of the exact value given, since it was near the limit of sensitivity of the measuring instrument used, and reading it required estimation of half a scale division. Numerous checks in which the number of filtering sheets and the lamp distance were varied without reference to previous settings seemed to indicate, however, that, whatever its absolute value, this intensity was at least as constant as any of the others.

the fish more constant, for all fish lost more or less pigment under laboratory conditions, and most of this loss occurred during the preliminary period. One of the unavoidable results of the necessity of keeping the fish for such a long time, however, was that all experiments were performed with individuals which were relatively pale and poor in pigment.

In the first series of experiments the fish were first placed for 1 hour on a white background under an illumination of 10 foot-candles and then transferred to black background at the test intensity. Observations were then made, as already described. The results obtained with 11 individuals are shown in the columns headed "A" in Table 1. The figures represent the limits of time in minutes within which change was judged to have been completed. From three to eight or ten readings were made to establish each limit, depending on the difficulties of judgment in the individual case.

TABLE 1

TIME (IN MINUTES) REQUIRED FOR CHANGE FROM LIGHT TO DARK CONDITION

A = after adaptation for 1 hour on white background at illumination of 10 foot-candles; B = after adaptation for 1 hour on white background at same illumination as test.

Fish No.	A 100 Ft.-C.	A 10 Ft.-C.	A 1 Ft.-C.	A 0.1 Ft.-C.	B 0.1 Ft.-C.	A 0.01 Ft.-C.	B 0.01 Ft.-C.
1	9–10	8– 9	8–10	18–20	30–35?	Inc. 120	Inc. 120
2	8–10	8–10	8– 9	12–15	8–10	25–30	9–10
3	8–10	8–10	8–10	15–18	8–10	25–30	8–10
4	10–15	9–10	8–12	18–20	14–15	30–35	9–10
7	14–15	14–15	16–18	Inc. 120*	Inc. 120	Inc. 120	Inc. 120
11	9–14	9–10	10–14	Inc. 120	Inc. 120	Inc. 120	Inc. 120
13	4– 6	3– 4	4– 5	Inc. 120	Inc. 120	Inc. 120	Inc. 120
14	4– 6	4– 6	4– 6	Inc. 120	Inc. 120	Inc. 120	Inc. 120
22	8–12	10–12	9–10	12–15	10–12	25–30	18–20
23	4– 6	3– 4	4– 6	18–20	6– 8	25–30	4– 5
24	4– 6	4– 6	4– 5	18–25	3– 4	18–20	3– 4
Av.	7.5–10	7.3– 8.7	7.5– 9.5	16–19	8.1– 9.8	25–29	8.5– 9.8

* Inc. 120 = change definitely incomplete after 120 minutes.

The average time required for change by the 11 fish was between 7 and 10 minutes at all three of the higher intensities—100, 10, and 1 foot-candles. A definite increase in the time required for change was seen at 0.1 foot-candle, and a further increase at 0.01 foot-candle. It is important to note that 4 fish definitely failed to turn completely dark even in 2 hours at 0.1 foot-candle while 5 failed to do so at 0.01 foot-candle. The significance of this will be discussed later. In the light of later results and in view of the work of Brown (1936), it now seems certain that the others were also incompletely dark at these intensities; but at least the condition of their melanophores was sufficiently close to complete expansion to be nearly indistinguishable by gross observation in the absence of a satisfactory standard of comparison.

In attempting to discover what was involved in the increase of reaction time at low intensity, a second series of readings at the two lower intensities was made. In this case the fish were first adapted for 1 hour on a white background at the same intensity for which their adaptation time on black was determined. The results are shown in the two

columns marked "B" in Table 1. The fish which had not turned completely dark under the previous experimental conditions still did not do so. The lengthening of adaption time previously shown by the others at intensities below 1 foot-candle was, however, definitely reduced. Fish No. 1 was an apparent exception, having required an even longer time to change than before; but since it died shortly afterward, this difference was probably due to some change in its physiological condition.

These results thus show that, when the fish were preadapted on a white background at 10 foot-candles, the time required for change to the dark condition was approximately constant at intensities of 1 foot-candle or more. At lower intensities, two changes in response appeared: first, a lengthening of adaptation time; and second, a tendency toward incomplete change. With preadaptation at the test intensity, the former tended to be reduced, the latter was unchanged.

TABLE 2

TIME (IN MINUTES) REQUIRED FOR CHANGE FROM DARK TO LIGHT CONDITION

A = after adaptation for 1 hour on black background at an illumination of 10 foot-candles; B = after adaptation for 1 hour on black background at the same illumination as the test.

Fish No.	A 100 Ft.-C.	A 10 Ft.-C.	A 1 Ft.-C.	A 0.1 Ft.-C.	B 0.1 Ft.-C.	A 0.01 Ft.-C.	B 0.01 Ft.-C.
27	1½–2	1½–2	1 –1½	2 –3	1¼–1¾	6 – 7	1 –1¾
28	1½–1¾	1¼–1¾	1 –2	2 –3	1¼–1¾	7 – 8	1 –2
37	1 –1½	1 –1½	1 –1½	1 –1½	1 –1½	7 –11	1 –1½
38	1 –1½	1 –1½	1 –1½	2 –3	1 –1½	4 – 6	1 –1½
26	1¾–2¼	1½–2	1½–2	1½–2	.....	1¼– 1¾	1½–2
36	.....	1 –1½	.....	1 –1½	.....	1 – 1½	¾–1¼
40	.....	2 –2½	.....	.....	.....	3 – 4	1½–2½
44	.....	1 –1½	.....	.....	.....	4 – 5	1 –1½
45	.....	1 –1½	.....	.....	.....	5 – 6	1 –1½
48	.....	1 –1½	.....	.....	.....	8 –10	1 –1½
Av.	1.4–1.8	1.2–1.7	1.1–1.7	1.6–2.3	1.1–1.6	4.6– 6.0	1.1–1.7

For a complete study of the problem, it seemed desirable to study adaptation in the reverse direction, namely, from dark to pale condition. It was impossible to complete this study with the same animals, but the results of a corresponding series obtained with other individuals appear in Table 2. Because of deaths, only 4 of the 10 fish represented could be followed through completely at all intensities; but these, as well as the other 6 on which determinations were completed at a few intensities, show results similar to those for the change from white to black. The lengthening of adaptation time at low intensities seemed to disappear in all cases where preparatory adaptation was made at the test intensity. The principal differences from the previous series of experiments were, first, that no animals definitely failed to undergo complete change at low intensities, as far as could be determined under the conditions of the experiment, and, second, that the time required for change was much shorter.

Thus, on a given background, either white or black, the rate of melanophore change was approximately constant at all of the higher intensities. The time required for change definitely increased at low intensity, however, unless previous adaptation had occurred

at the same low intensity. The fact that this increase in the time required for change apparently tended to disappear when the fish were exposed to the same low intensity throughout the experiment might suggest that the factor responsible for the longer adaptation time originally observed was the time required for the adaptation of the eyes to the low illumination. But while this factor may be important, it cannot explain the fact that such a large proportion of the fish definitely failed to turn completely dark at low illumination under any conditions.

A possible clue as to what occurred was offered by the observation that at low intensities, on both white and black backgrounds, it seemed much more difficult than at higher intensities to determine whether complete change had taken place. From this, together with the observed definite failure of a large proportion of the fish to turn completely dark under low illumination, the suspicion arose that the condition on either white or black background under low illumination was an equilibrium short of complete contraction or expansion of the melanophores.

Direct evidence for incomplete change at low intensities was obtained by adapting 5 normal minnows each on white and black background at 0.01 foot-candle for 1 hour, then throwing them into hot formalin solution and comparing pieces of the integument with similar pieces from minnows which had been on similar backgrounds at an illumination of 10 foot-candles for the same length of time. Because of the small number of fish available and the great variation in the size and form of the melanophores, measurement of the melanin masses or other attempts to make strictly quantitative comparisons seemed unlikely to offer any advantage. Conclusions were, therefore, reached by comparing two pieces of integument during several minutes of study and recording the general impression obtained. The conclusions reached were checked in each case by persons who knew nothing of the source of the pieces or the nature of the experiment but were simply asked to decide whether or not there was greater pigment dispersion in one case than another. In no case was there any doubt that under low illumination greater dispersion had occurred on a white background, and less dispersion on a black one, than in the controls.

Thus, neither complete dispersion nor complete contraction of the pigment occurs at low light intensity. It follows, therefore, that under such low intensity the observed time for color change is the time required for change from a partly dark to a partly light condition or the reverse. Consequently, even though the observed adaptation time be as short under some conditions as at higher intensity, the rate of change must have decreased because of the corresponding decrease in the amount of change.

In this connection the response of the fish to complete darkness is also of interest. Careful observations at various times on some thirty specimens of *Nocomis* have shown that in the dark a clearly intermediate condition is gradually reached, regardless of whether the fish are dark or pale to begin with. While exact determinations were not made, the minimum time for a reasonably complete approach to the equilibrium condition in darkness seemed to be about $\frac{1}{2}$–1 hour. The condition, once attained, persisted for at least 24 hours, the longest time the fish were left in the dark, so that this response to darkness is not a transient phenomenon. Various observations on other minnows, such as *Semotilus atromaculatus* and *Pimephales promelas*, have given similar results.

It appears, therefore, that the melanophore change at the lower intensities of illumination approaches the change which occurs in darkness in both rate and amount. In the dark the melanophores slowly reach an intermediate condition of expansion. Under low

illumination the time required for change, while less than that required in the dark, is greater than that required in stronger light. Likewise, though the response to background is still dominant even at the lowest intensity used, the expansion of the melanophores is less on black, and greater on white, background than it is at higher intensities.

The photic processes involved in melanophore changes at low light intensity must be limited by the sensitivity of the receptive apparatus. It is interesting to note that Brown (1936), in experiments published shortly after the completion of the foregoing, found 1.75 foot-candles to be the lower limit at which complete melanophore expansion would occur in *Ericymba buccata*, and that below this point expansion was proportional to the intensity. In my own experiments, approximately complete melanophore change at a constant rate appeared to occur at and above about 1 foot-candle. Thus, changes appear to be independent of intensity down to about the same point in both of these minnows. It seems conceivable that the point where melanophore activity becomes dependent on light intensity may be the point where cone vision ceases. It must be borne in mind, however, that fish without eyes, though they no longer respond to background, may still show melanophore responses to changes in illumination, so that the problem of the exact structures and processes involved in reception is not a simple one.

To what extent receptors other than the eyes were involved in the present results is not certain. The effect of light as such is always an expansion of the melanophores. It would appear, therefore, that a fish on either black or white background should be slightly darker if the direct response to light were involved than would otherwise be the case, since the effect of a black background would be reinforced while that of a white background would be opposed. Whether this is true could be determined only by eliminating the direct stimulation without affecting vision, and no convenient way of doing this has been found. Illumination of the eyes alone is almost impossible as long as the fish are free to move, while any restraint of the fish results in an excitement which itself has very marked effects on the condition of the melanophores.

It is, however, a relatively simple matter to remove the eyeballs and thus eliminate the response to background. Tests with 12 fish thus blinded showed that the melanophore change of which they were capable in response to differences in illumination was, at best, not more than half as great as that of normal fish. Such blinded fish appeared to be maximally dark at light intensities of 1 or 2 foot-candles or more. Reduction of the light produced a partial melanophore contraction, so that on a black background at intensities of 0.1 and 0.01 foot-candle the blinded fish were usually less dark than normal ones. Even in the complete absence of light, however, the appearance of the blinded fish could best be described as "moderately dark." Normal fish on a white background, even at 0.01 foot-candle, were much more pale than any blinded fish was ever observed to become, and also more pale than they themselves would be in complete darkness. Thus, although the observed responses of normal fish may have been the result of several stimuli, it at least seems clear that the response to background was predominant even at the lowest intensity used.

It may still be asked why the low rate of change which does occur at low light intensity does not eventually result in the same amount of change as the rate prevailing at higher intensity, even though the time to reach the final condition might be longer. The answer probably is that, while photic stimulation ordinarily dominates and determines the condition of the melanophores, it is not the only force capable of affecting them. In the complete absence of light, melanophores may still change as a result of these other

forces. Below a certain point, the influence of light is no longer completely dominant, and the condition of the melanophores is then determined by the sum of all the forces of the external and internal environment which are capable of affecting them, and perhaps by the most stable condition inherent in the structure of the melanophores themselves. The weaker the light, then, the more closely will the equilibrium condition of the melanophores approach the one attained under similar conditions in the complete absence of light, and the more closely will the time required for the change to this condition approach the time required for the corresponding change in the absence of light.

SUMMARY

The melanophore responses of *N. biguttatus* to white or black background with illumination from above have been found to be approximately constant in rate and amount at light intensities of 100, 10, and 1 foot-candle.

A decrease in both the rate and amount of melanophore change appeared at an intensity of 0.1 foot-candle, and a further such decrease at 0.01 foot-candle. These changed responses are thought to be due to the incomplete stimulation of the visual elements by such low illumination.

Although the influence of the background still appeared to be most important, the response on either white or black background at these low intensities began to approach the response to complete darkness, namely, a slow change to a condition of partial expansion of the melanophores.

LITERATURE CITED

BROWN, F. A., JR. 1936. Light intensity and melanophore response in the minnow, *Ericymba buccata* Cope. Biol. Bull., **70**:8–15.

DUSPIVA, FRANZ. 1931. Beiträge zur Physiologie der Melanophoren von Fischembryonen. Sitz.-ber., Akad. d. Wiss., Wien, mathem.-naturw. Kl., **140**:553–96.

KOLLER, G. 1934. Über den Farbwechsel von Coregonenlarven. Biol. Centralbl., **54**:419–36.

MAST, S. O. 1914. Changes in shade and pattern in fishes and their bearing on the problems of adaptation and behavior. Bull. U.S. Bur. Fisheries, **34**:177–238.

MURISIER, P., 1921. Le Pigment mélanique de la truite. Rev. suisse de zool., **28**:45–98, 149–96, 243–300.

POUCHET, G. 1876. Des changements de coloration sous l'influence des nerfs. Jour. anat. et physiol., **12**:1–90, 113–65.

SUMNER, F. B. 1911. The adjustment of flatfishes to various backgrounds. Jour. Exper. Zoöl., **10**:409–505.

THE SIMILARITY BETWEEN THE HYPOPHYSEAL CHROMATOPHOROTROPIC HORMONE AND THE CHROMATOPHOROTROPIC HORMONE OF THE CRUSTACEAN EYESTALK

(One plate)

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THE discovery of Smith (1916) and Allen (1916) that extirpation of the hypophyseal anlage in embryos of the tadpole was followed by striking changes in the pigmentary coat of the developing animals led to a complete revision of the problem of chromatic activity. This, and especially the subsequent work of Atwell (1919), Swingle (1921), Krogh (1922), and Hogben (1924), demonstrated quite convincingly that the pituitary gland secretes a hormone which controls specifically the expansion of the integumentary melanophores of the amphibians, and thus delivered the first blow to Pouchet's conception (1876), which was generally held prior to 1915, that melanophore reactions of animals were governed through the intervention of peripheral nerves. Since this date it was found in rapid succession that the chromatophores of crustaceans (Perkins, 1928; Koller, 1928), most elasmobranchs (Lundstrom and Bard, 1932), cyclostomes (Young, 1935), and some reptiles (Noble and Bradley, 1933; Kleinholz, 1935) were controlled by hormones.

The teleosts and possibly also the African chameleons (although the latter have not yet been investigated) do not lose completely their ability to respond to black backgrounds following hypophysectomy (von Frisch, 1911; Desmond, 1924; Matthews, 1933; Parker, 1934; Abramowitz, 1936*a*, 1937*d*). The comparative physiology of these two groups whose melanophores appear to be controlled chiefly by the autonomic nervous system has been discussed in detail by Sand (1935), but there has been no similar comparative treatment of the melanophore reactions of those animals whose color changes are due directly to the intervention of blood-borne hormones. The experiments and results which have been published in abstract form elsewhere (Abramowitz, 1936*b*, *c*, *d*) form the foundation for a comparative treatment of the endocrine basis of pigmentary activity in the crustaceans and the lower vertebrates, and serve to point out certain similarities between the chromatophorotropic hormones of these two groups. Professor Parker's book (1936), which integrates in terms of neurohumoralism the older concepts of nervous and endocrine mechanisms relating to chromatophores, is especially valuable for a unified concept of the entire field of chromatic physiology.

MATERIALS

1. *The chromatophores.*—Lower vertebrates possess four kinds of chromatophores which are single cells and are always monochromatic. These are the melanophores, containing melanin; guanophores, containing pigments derived from the purines; xanthophores and erythrophores, whose pigments belong to the carotenoid series. Crustacean

chromatophores, however, are not usually single cells but are composed of two or more cells, somewhat like a coenocyte (Keeble and Gamble, 1904). Furthermore, crustacean chromatophores may be monochromatic, like those of the vertebrates, or polychromatic, in which case two or more pigments are present within the same chromatophore. Chemically, pigments similar to those mentioned occur in the crustaceans. However, the pigments of the Crustacea cannot be positively identified by their colors, for the melanins range from yellow through red to black, while the polyene pigments also range from yellow to dark brown in color. Consequently, certain crustacean chromatophores which have been designated as melanophores may in reality be "carotenophores," and vice versa.

It appears that the chromatophores of the vertebrates may be compared only with the monochromatic chromatophores of the Crustacea, if with any at all, and that only chromatophores containing similar pigments may be safely compared with one another. Thus, the melanophores of vertebrates may be compared with monochromatic melanophores of crustaceans, the xanthophores with the monochromatic chromatophores containing a similar pigment in the crustaceans, etc. The polychromatic chromatophores of the crustaceans, it appears, must be treated as a special type and cannot be compared fairly with any chromatophore of the vertebrates, for nothing anatomically similar to these polychromatic chromatophores occurs in the vertebrates. Whenever melanophores occur abundantly in either the crustaceans or the vertebrates, they are usually the chief instruments in provoking color changes. In this paper the melanophores were studied almost exclusively in the brachyuran crustaceans and in such vertebrates as the frog, the lizard, the dogfish, and several teleosts.

2. *The hormones.*—The vertebrate chromatophorotropic principle is found in the pituitary gland from cyclostomes through primates. It has been termed the "melanophore hormone" (Smith and Graeser, 1924; Jores, 1932; Dietel, 1932; Stehle, 1936; to mention a few), the " 'B' substance" (Hogben and Slome, 1936), and "intermedin" (Zondek and Krohn, 1932*a*). This principle affects not only vertebrate melanophores (Hogben, 1924) but also erythrophores (Abolin, 1925; Saphir, 1934; Zondek and Krohn, 1932*a*, *b*) and xanthophores (Abolin, 1925; Giersberg, 1932; Abramowitz, 1936*e*). The possibility that there are separate hormones for each of these three chromatophores exists (cf. editor's footnote, Zondek, 1935*a*, *b*), and, in fact, the separate identities of a melanophore and an erythrophore hormone have been advocated (Jores and Lenssen, 1933; Rodewald, 1935). This has, however, been denied by Böttger (1934, 1936). Consequently, it will be perhaps best, for the present, to regard the chromatophorotropic principle as a hormone which affects all three chromatophores, until it is shown conclusively that more than one hormone is involved. The most suitable of the names for this hormone, therefore, is "intermedin," since this term does not necessarily refer to an effect on any particular type of chromatophore.

The relation of intermedin to other hypophyseal principles seems to be clearly indicated. Zondek and Krohn (1932*d*) claimed their intermedin had no anterior or posterior lobe hormones, but antidiuretic activity has been found (Sulzberger, 1933; Turner, 1935). Downes and Richards (1935) achieved a partial separation of antidiuretin and intermedin. Finally, Fisher (1936) reported that atrophic pars nervosa lacks vasopressin, antidiuretin, and oxytocin, but not the chromatophore factor. It appears, therefore, that intermedin is a distinct hormone of the hypophysis. However, the vasopressor and antidiuretic effects may be due to one hormone (Fraser, 1937).

The hormone of the crustacean eyestalk has been called the "eyestalk hormone" (Perkins, 1928), the "crustacean chromatophore activator" (Kropp and Perkins, 1933), and the "crustacean chromatophorotropic hormone" (Abramowitz, 1937*b*). All these general terms are applicable because the eyestalk extract affects the erythrophores, melanophores, guanophores, and "xanthophores" of the Crustacea. Neither vertebrate nor invertebrate pigmentary hormone has been isolated in chemically pure form.

The terms "intermedin" and "eyestalk hormone" will be used throughout this paper. All experiments with intermedin were performed with dilutions of one stock solution prepared from commercial sheep (whole gland) pituitary powder. Oxytocic and vasopressor activity are negative, but some antidiuretic effect was found by testing normal frogs. Quantitative treatment is obtained by expressing the amount of intermedin injected in terms of gram equivalents of the powder. Eyestalk hormone was prepared as a water extract of the eyestalks, boiled to coagulate the proteins, or, in some cases, in more purified form (Abramowitz, 1936*b*).

EXPERIMENTS AND METHODS

I. THE COMPARATIVE PHYSIOLOGY OF MELANOPHORE RESPONSES; THE RELATIONS AMONG THE EYES, ENDOCRINE GLANDS, AND THE MELANOPHORES

All investigators are agreed that the eyes are essential in initiating melanophore responses of animals, such as are induced by placing the animal over illuminated white or black backgrounds. Yet the relation between the eyes and the pituitary in the vertebrates and the eyes and the eyestalk glands of the Crustacea has not been definitely solved. This relationship can be analyzed by performing similar surgical operations. When the eyes are removed from such representative specimens as the frog, the lizard, the dogfish, and the lamprey, the melanophores of the integument become expanded and remain in a more or less expanded state indefinitely, provided illumination is present and the temperature is maintained normal for each particular specimen. When a similar operation is performed on the eyestalks of the brachyurans by excision of only the retina, the same result is obtained, although in this case the existence of a diurnal rhythm modifies the result (Abramowitz, 1937*b*). The expansion of the melanophores following excision of the retina has been thought by Parker, Brown, and Odiorne (1935) to be due to an innate property of these chromatophores. However, this does not seem to be valid, because, if such blinded vertebrates are now hypophysectomized, complete and enduring pigment concentration ensues. Similarly, if the remainder of the eyestalks in which the endocrine glands are located is excised, the melanophores of the brachyurans become and remain contracted. Thus, it seems that, when the eyes are removed in the vertebrates, the pituitary secretes intermedin continuously under the specified environmental conditions because a mechanism (eyes) which can completely inhibit the release of the hormone is removed.[1] A similar situation is found in the brachyurans, where excision of the retina produces a continuous secretion of the chromatophorotropic hormone for 3 or 4 days before the diurnal rhythm is re-established. The entire situation is summarized in Tables 1 and 2.

This similarity in the behavior of melanophores was pointed out by the author (1935),

[1] In the cyclostomes, the pineal complex supplements the eyes in inhibiting the release of melanophore hormone (Young, 1935). In this connection it might be pointed out that a light receptor in the skin may aid in keeping blinded animals dark by stimulating the pituitary.

and led to the studies now being reported. It seems clear that the reactions of the melanophores of the Brachyura and of the vertebrates as listed in Table 1 are identical following the removal of the chromatophorotropic hormone from the circulation. Administration of the hypophyseal chromatophore hormone easily restores the dark coloration in hypophysectomized frogs (Hogben, 1924), lizards (Kleinholz, 1935), dogfishes (Lundstrom and Bard, 1932), and lampreys (Young, 1935). Administration of the eye-

TABLE 1

CONDITION OF MELANOPHORES AFTER REMOVAL OF THE EYES AND GLANDS

Author	Species or Genus	Condition	State of Melanophores
Megašur, 1912	*Uca pugnax*	Eyestalks extirpated (glands and eyes removed)	C
This paper	*U. minax*		C — Invertebrates
Abramowitz, 1935	*Portunus anceps*		C — Brachyura
Carlson, 1935	*U. pugilator*		C
This paper	*Mustelus* (pups)	Hypophysectomized +eyeless	C — Vertebrates Elasmobranch
This paper	*Rana*		C — Amphibian
Kleinholz*	*Anolis*		C — Reptile
Hogben and Slome, 1931	*Xenopus*		C — Amphibian
Young, 1935	*Lampetra*		C — Cyclostome

Symbols: C = concentrated; * = personal communication.

TABLE 2

SUMMARY OF THE EFFECTS OF GLAND OR EYE REMOVAL

Animal	Condition with Illumination and Normal Temperature	State of Melanophores
Vertebrates	Eyes and hypophysis removed	C
	Eyes intact, hypophysis removed	C
	Eyes removed, hypophysis intact	E
Invertebrates (Brachyura)	Retina and glands removed (eyestalk extirpation)	C
	Retina intact, glands removed	?
	Retina removed, glands intact	E

Symbols: C = contracted; E = expanded; ? = has not been performed.

stalk hormone to blinded brachyurans also restores the dark coloration (Carlson, 1935; Abramowitz, 1936*c*). Thus, the responses of the melanophores of the brachyurans and of the vertebrates cited to the chromatophore hormone of each group, respectively, are also identical. The possibility that the identical reactions might be due to a similarity in the hormones involved was next investigated.

II. EFFECTS OF THE EYESTALK HORMONE ON VERTEBRATE CHROMATOPHORES

Koller and Meyer (1930) and Meyer (1931) showed that eyestalk extracts of *Crangon* contracted the melanophores of *Gobius* and *Pleuronectes*, while rostral organ extracts

expanded these cells. Perkins and Kropp (1932*a*, *b*) and Kropp and Perkins (1933) found that extracts of *Crago* produced melanophore contraction in *Anguilla*, *Myoxocephalus*, and *Limanda;* no response in *Pollachius* and *Scomber;* and melanophore expansion in *Clupea*. Extracts of the eyestalks of *Pagurus* and *Homarus* produced melanophore contraction in *Fundulus*. Furthermore, these authors found that stalk extracts of *Palaemonetes*, *Callinectes*, *Homarus*, or *Pagurus* produced melanophore expansion in white-adapted tadpoles.

1. *Amphibians.*—Kropp and Perkins' results on normal white-adapted frog tadpoles were first verified. Ten normal white-adapted tadpoles were injected intraperitoneally with an extract of *Palaemonetes* eyestalks. The injection volume was 0.1 cc., and the concentration of hormone was 50 E.S./cc.[2] Melanophore expansion occurred in 9 animals. Similar injections into normal black-adapted specimens brought about no change in the already expanded melanophores. About 30 hypophysectomized specimens[3] were injected, with results similar to those of the white-adapted group (Pl. I, Figs. 1 and 2). The normal tadpoles were maintained on the same background after making the injection as before, a procedure which is standard for all experiments in which normal animals are used.

The same experiment was repeated with adult frogs (*Rana pipiens*). One-half cubic centimeter of a purified extract of 100 E.S./cc. (*Palaemonetes*) was injected into the dorsal lymph sac. Five white-adapted, 5 black-adapted, and approximately 20 hypophysectomized frogs were tested, and the results described for the tadpoles were verified (Pl. I, Fig. 3). The injected pituitaryless specimens remained dark, in some cases, for 10 hours. Moreover, responses could be elicited with injections of as little as 10 or 15 E.S. (about 3 *Uca* units). Frog melanophores are exceedingly sensitive to intermedin, for darkening occurs in hypophysectomized specimens after injection of 0.01 mg. equivalent.

2. *Elasmobranchs.*—As test objects, the pups of the smooth dogfish, *Mustelus canis* were used. Extracts of the eyestalks of *Libinia* and *Callinectes* were tested. A dose of 20 E.S. was found to produce complete expansion of the melanophores of hypophysectomized pups (eight tests) when injected intramuscularly. Intraperitoneal injections were negative. The animals which responded positively were injected near the anterior dorsal fin, and the darkening that appeared usually enveloped most of the anterior half of the fish. The same dosage tested similarly on 4 normal black-adapted specimens produced no change in the expanded melanophores. The expansion of melanophores in the hypophysectomized series persisted for 5 hours. The dosage of the crustacean hormone is rather high (about 80 *Uca* units), but the minimum dosage of intermedin injected intraperitoneally necessary to darken hypophysectomized pups was found to be 0.1 mg. equivalent (20 animals tested).

3. *Reptiles.*—Unfortunately, only few experiments were performed, owing to difficulties encountered in securing the lizard, *Anolis*. Through the kindness of Dr. L. H. Kleinholz, 7 *Anolis* were available. Three were hypophysectomized by a direct oral route (one dying the next day); and 4 normal animals, 2 white-adapted, and 2 black-adapted, were used as controls. These specimens were injected intraperitoneally with

[2] The letters "E.S." are used as an abbreviation for eyestalk.

[3] Two-thirds of these hypophysectomized tadpoles were kindly given to me for these experiments by Dr. W. M. Etkin, of the American Museum of Natural History, New York, to whom I am much indebted. The remaining tadpoles were hypophysectomized at the 4-mm. stage by Allen's method (1916).

0.1 cc. of an extract of *Palaemonetes* in a concentration of 100 E.S./cc. The normal black-adapted specimens showed no changes. The normal white-adapted animals showed irregular dark areas (melanophore expansion) along the ventral surfaces, but the dorsal surface remained green (melanophore contraction). One of the hypophysectomized animals responded in a similar fashion, while the other showed a more uniform darkening (Pl. I, Fig. 4).

4. *Teleosts.*—The catfish, *Ameiurus*, and the killifish, *Fundulus*, were used as test objects. The catfishes were hypophysectomized by the opercular approach (Abramowitz, 1937*a*), and bands of caudal melanophores were denervated by Wyman's (1924) technique. One set of 6 animals was placed in white vessels for 5 days, and another set of equal number placed in black dishes. Two sets of normal animals were likewise placed in white or black dishes. Innervated and denervated melanophores of both normal and hypophysectomized specimens become adjusted to these backgrounds (Parker, 1934). In the white vessels, both innervated and denervated cells become contracted; while in black surroundings, the melanophores (innervated and denervated) of normal animals are fully expanded but those of hypophysectomized animals are not completely expanded (Abramowitz, 1936*a*).

Each animal was injected intraperitoneally with an extract of 100 E.S. of *Palaemonetes*, but the results were not clear cut. Within an hour there was no change in the black-adapted normal series, usually a slight expansion in the innervated and denervated areas of the white-adapted normal and hypophysectomized groups, and inconclusive changes in the black-adapted hypophysectomized group. The experiment was repeated by injecting a larger dose (200 E.S.) into 15 white-adapted normal fishes. Now the expansion of innervated and denervated cells was more marked; and a condition of half-expansion, remaining for 2 hours, was obtained in 7 animals. The remaining 8 animals darkened very strongly, but died during the experiment, since the excessive dose was undoubtedly toxic. The experiment was again repeated, using a more purified extract (Abramowitz, 1936*b*) and injecting a smaller dose intramuscularly in order to obtain a more local response. While this type of an injection is undoubtedly inferior, because subcutaneous injections in teleosts result often in nonspecific effects, it does avoid the use of a dose which is toxic. Accordingly, 24 fishes were divided into four groups as described above, with the exception that bands of cells were not denervated. Injections of 50 E.S. (*Palaemonetes*) were made into the dorsal muscles at a region slightly anterior to the anterior dorsal fin. After 15–20 minutes, melanophore expansion resulted in the two white-adapted groups and in the black-adapted hypophysectomized group but no change occurred in the normal black-adapted group.

It is difficult to conclude definitely that the eyestalk extract expands the melanophores of the catfish, since the necessary proof (complete expansion in a denervated area) was not obtained. At best, the denervated cells showed a condition of half-expansion. The reaction of the innervated cells might be due actually to a toxic action on the nervous system, though this could not account for the response of the denervated area. Repetition of this experiment with hypophysectomized *Fundulus*, a section of whose tails was denervated by Wyman's method, gave negative results. In no case did the crustacean extract, when injected intraperitoneally, produce melanophore reactions in either innervated or denervated melanophores.

The expansion of erythrophores of teleosts, such as *Phoxinus*, is claimed by Zondek and Krohn (1932*a*, *b*) to be a specific indication of the presence of intermedin. Although

this is questionable, it was of interest to test the action of the crustacean hormone upon the erythrophores. No *Phoxinus* being available, the dace, *Chrosomus erythrogaster*, was used (Saphir, 1934; A. Young, 1934). Twenty-four fish were injected either subcutaneously or intraperitoneally. The injection volume was 0.1 cc. of a stock solution of 100 E.S./cc. (*Palaemonetes*). Seventy per cent of the animals injected subcutaneously showed erythrophore expansion (some also showed melanophore expansion), but intraperitoneal injections were rarely followed by a positive response.

Taking all of the results into consideration, it appears that the action of the crustacean extract on vertebrate melanophores is to induce generally the expanded state of these chromatophores. This action is characteristic of intermedin, and it seems that the effect of these two extracts on the melanophores is similar. Furthermore, the erythrophores of vertebrates tend to respond similarly. Information concerning the action of the crustacean hormone on vertebrate xanthophores would be exceedingly interesting.

III. EFFECT OF INTERMEDIN ON CRUSTACEAN CHROMATOPHORES

The brachyurans, *Uca pugilator* and *U. pugnax*, were chosen as test objects (Abramowitz, 1936*c*). Sixty animals, whose eyestalks were extirpated 2 days previously, were injected with various known doses of intermedin. These blinded animals are pale, regardless of light intensity or of background. The most suitable dose was found to be 0.05 cc. of a concentration of 1 gm. equivalent of intermedin in 1 cc. of sea water. Typical responses ensued (cf. Abramowitz, 1937*c*). The animals became dark within $1\frac{1}{2}$ hours, remained dark for 7 hours, and finally became pale again (Pl. I, Figs. 5 and 6). Microscopic examination disclosed that intermedin produced expansion of the melanophores and contraction of the guanophores and "xanthophores." The erythrophores were unaffected, these remaining contracted. The eyestalk hormone of *Uca* (or of any other crustacean tested [cf. Abramowitz, 1937*c*]), when injected into blinded specimens, produces the same reaction except that the erythrophores become expanded. Thus, there is a significant difference between the qualitative effects of vertebrate and invertebrate pigmentary extracts in the crab, *Uca*.

IV. EFFECT OF EXTRACTS OF THE ASCIDIAN SUBNEURAL GLAND COMPLEX ON VERTEBRATE AND CRUSTACEAN MELANOPHORES

Because of its embryological history and cytological appearance, the subneural gland has been considered a primitive pituitary gland. Butcher (1930) demonstrated that the subneural gland contains the oxytocic hormone, and Bacq and Florkin (1935) and Abramowitz (1937*c*) showed that the chromatophorotropic principle is also present. The former produced melanophore expansion in normal frogs with an extract of *Ciona*, while the latter produced melanophore expansion in *Uca* with an extract of *Molgula* (subneural gland = 0.2 *Uca* units). The vasopressor hormone is also present in this primitive pituitary of protochordates (Bacq and Florkin); and recently Hogg (1937) has reported a gonadotropic effect of the subneural gland of *Polycarpa*, the large Bermudan form, on the immature mouse.

V. COMPARATIVE PROPERTIES OF THE CRUSTACEAN HORMONE AND INTERMEDIN

The properties of the two hormones may best be compared in tabular form (Table 3).

Intermedin is potentiated from ten to twenty-five times by exposure to alkali (Hogben and Winton, 1922; Smith and Graeser, 1924; Zondek and Krohn, 1932*d*; Böttger, 1934;

Jores, 1934; Stehle, 1936). The eyestalk hormone is stable to NaOH when a mixture of the hormone and NaOH is boiled for 5 minutes (Carlson, 1936) but not for longer periods (Abramowitz, 1937*b*). If the potentiation by alkali is due to a change in the intermedin molecule (Stehle, 1936), it would seem that there is a significant chemical difference between the invertebrate and vertebrate hormones. However, the potentiation may be due to the removal of an inhibiting substance (Böttger, 1936) or to circulatory facilitation resulting from the destruction of vasopressin by alkali (Hogben and Gordon, 1930).

DISCUSSION

The general trend of the evidence seems, therefore, to indicate a similarity between the vertebrate and invertebrate pigmentary hormones. This similarity between the eyestalk hormone and "pituitary secretion" was first pointed out by Kropp (1932), inasmuch as both eyestalk extracts and pituitrin expanded tadpole melanophores. Additional evidence has been presented in this paper from the comparative physiology of melanophore responses, from some chemical and physical properties of the two hormones, and from reciprocal replacement therapy tests. Nevertheless, while the similarity seems to be striking in several respects, one must approach the problem with a good deal of caution.

TABLE 3

	Crustacean Eyestalk Hormone (Abramowitz, 1937*b*)	Intermedin (Zondek and Krohn, 1932*d*)
Water	Soluble	Soluble
Ethanol (absolute)	Soluble	Soluble
Ethyl ether	Insoluble	Insoluble
Chloroform	Insoluble	Insoluble (practically)
Acetone (absolute)	Insoluble	Insoluble
Benzine	Insoluble	Insoluble
Methanol (absolute)	Soluble	Soluble
Acid (HCl)	Stable	Stable
Alkali (NaOH)	Stable	Stable
Boiling	Stable	Stable
Oxidation	Destroyed	Destroyed

In the first place, the evidence from the comparative physiology of melanophores may be a peculiar coincidence whose significance is quite different from that herein described. Two significant exceptions must be noted. The black pigment (a melanin) of *Crangon* expands when the eyestalk glands are removed (Koller, 1930), and contracts when the animal is injected with the eyestalk hormone. However, it becomes dispersed when *Crangon* is injected with hypophysin (Böttger, 1934). Thus, the two extracts produce opposed reactions. The full significance of these observations cannot be fully appreciated at present because the black pigment of *Crangon* is contained mainly in polychromatic chromatophores. The other case is that of *Ligia* (Kleinholz, 1937). *Ligia* has monochromatic melanophores (single cells!) which contract when its hormone is injected. This is, of course, opposite to the behavior of the melanophores of the brachyurans listed in Table 1. The action of intermedin or of the brachyuran eyestalk on *Ligia*'s melanophores would be well worth investigating in this connection.

The reliability of the reciprocal replacement therapy experiments is undermined by the existence of nonspecific responses of the melanophores. This danger, one of the greatest in chromatophore research, has not been duly emphasized, for chromatophores are unusually sensitive structures and may be induced to respond to an array of chemical and physical stimuli. It is generally acknowledged that intermedin is a specific hormone for vertebrate chromatophores. Extracts of tissues other than the pituitary gland do not produce melanophore expansion in a hypophysectomized specimen (Hogben, 1924). The same may also be true of the brachyuran and other crustaceans with respect to the eyestalk glands. Yet the possibility exists that the eyestalk hormone produces only a nonspecific response when injected into a hypophysectomized vertebrate, and vice versa. This question cannot be definitely answered at present, for there is still little known concerning the existence of nonspecific melanophore responses in hypophysectomized vertebrates or eyestalk-amputated brachyurans.[4]

The qualitative differences, already mentioned, in the reciprocal actions of the pigmentary hormones cannot be properly explained. The failure of intermedin to expand the erythrophores of the crab, *Uca*, should be investigated further, and the reactions of the melanophores in *Crangon* are still uncertain. Consequently, these differences cannot be discussed adequately until more observations are at hand. From the quantitative standpoint, only the response of the hypophysectomized frog to eyestalk extract is similar to that resulting from a weak dosage of intermedin. The action of intermedin in *Uca* in the concentration used is indistinguishable quantitatively from that of an eyestalk extract containing 2 *Uca* units. However, this dose of intermedin is significantly high. Greater dilutions usually produced no reactions; furthermore, I have been unable to balance comparable doses by testing frogs and crabs simultaneously. In other words, 0.05 gm. equivalents of intermedin are quantitatively equal in effect on the crab to 2 *Uca* units, but 2 *Uca* units of *Palaemonetes* hormone are ineffective usually on the hypophysectomized frog, while 0.05 gm. equivalents of intermedin will darken frogs for several days. Much more pituitary hormone is needed, then, to produce a reaction in crabs than in frogs, and vice versa. Assuming that the reciprocal effects are due to the hormones and are not results of toxicity or contamination of the extracts, the explanation of this difference may be: (1) a difference in the responsive tissues, since a crustacean melanophore and a vertebrate melanophore may be as different physiologically as they are anatomically, (2) a difference in the chemical composition of the hormones or impurities present in the extract.[5]

Concerning the first, it can be pointed out that the melanophores of *Uca* do not con-

[4] Another point to be considered is the problem of the number of pituitary chromatophorotropic hormones. The recent work of Hogben (1936) and of Hogben and Slome (1936) points to two pituitary pigmentary hormones—an expanding (intermedin, "B" substance) and a contracting ("W" substance) hormone. The previous discussion has proceeded on the assumption that only one hormone (intermedin) is involved in the chromatic physiology of amphibians and other vertebrates, simply because the bulk of this work had been completed before the most recent papers of Hogben and of Hogben and Slome had appeared. Moreover, the interpretations of most of the data, already presented, would not be seriously invalidated by the existence of the "W" hormone, since only the expanding hormone is under discussion.

[5] A third possibility is that both the hormones and the melanophores of brachyurans and vertebrates are different but that the mechanism by which intermedin expands a vertebrate melanophore may be similar to that by which the eyestalk hormone expands a crustacean melanophore. There is, however, nothing known concerning the microphysiology of the melanophores under endocrine influences.

tract to adrenalin, while those of fish and amphibians, as is well known, react to very small doses. Furthermore, the melanophores of some selachians are refractive to adrenalin, while in other elasmobranchs adrenalin may produce pallor indirectly, through vasoconstriction (Wykes, 1936). It is obvious, then, that vertebrate melanophores do not always react by contracting to adrenalin hydrochloride; and, indeed, the reaction, at least in one particular case (*Anolis* [Kleinholz, 1936]), may be just the reverse. Vertebrate melanophores, therefore, may be slightly different physiologically from one genus to another.

The second possibility cannot be adequately discussed because neither of the two hormones has been obtained in a pure state. The word "similarity" has been used throughout this paper to mean a similarity in physiological effect, not a chemical similarity or identity. The activity of both is found in more or less the same solvents, and some other properties are common to both; consequently, on this basis they may be said to behave alike. Judging from the chemistry and physiological effects of the sex hormones, for example, it would be extremely surprising to find chemically identical pigmentary hormones in the crustacean eyestalk, the ascidian subneural gland, and the vertebrate pituitary. Intermedin may even be slightly different chemically in the pituitary of different vertebrates. What may be possible, however, is that the vertebrate and invertebrate hormones may be related sufficiently to produce reciprocal pigmentary reactions when given in proper doses.[6]

SUMMARY

The monochromatic melanophores of the brachyurans remain expanded after loss of the eyes but contract after removal of the eyestalks. These responses are similar to those of the melanophores of amphibians, reptiles, elasmobranchs, and cyclostomes following optic enucleation and hypophysectomy, respectively. By reciprocal replacement therapy eyestalk extracts of several crustaceans have been shown to be effective in expanding melanophores of an elasmobranch (*Mustelus*), a teleost (*Ameiurus*), an amphibian (*Rana*), and a reptile (*Anolis*). Similarly, intermedin, the hypophyseal hormone, was found to produce an expansion of the melanophores of eyestalk-amputated brachyurans (*Uca*). The comparative chemical properties of the two pigmentary hormones are listed and discussed.

LITERATURE CITED

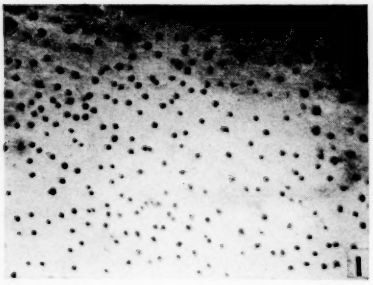
ABOLIN, L. 1925. Arch. f. mikr. Anat., **104**:667.
ABRAMOWITZ, A. A. 1935. Proc. Nat. Acad. Sci., **21**:677.
———. 1936*a*. Biol. Bull., **71**:259.
———. 1936*b*. Proc. Soc. Exper. Biol. and Med., **34**:714.
———. 1936*c*. Proc. Nat. Acad. Sci., **22**:521.
———. 1936*d*. Anat. Rec., **67**:108.

[6] Whether or not crustaceans other than brachyurans will respond to intermedin is not fully known. Dr. Kleinholz and I have found that intermedin contracts the red and yellow pigments of *Palaemonetes* in approximately 50 per cent of the cases. Furthermore, intermedin did not affect the retinal pigments of *Palaemonetes*. Eyestalk extracts, however, affect both body and retinal pigments of *Palaemonetes*. Consequently, *Uca* may be peculiar among crustaceans in that it responds definitely to intermedin preparations. This is an additional point which indicates that the similarity between the pigmentary hormones may not only be limited to several properties but may also be limited in scope to certain animal groups.

ABRAMOWITZ, A. A. 1936*e*. Amer. Nat., **70**:372.
———. 1937*a*. Science, **85**:609.
———. 1937*b*. Biol. Bull., **72**:344.
———. 1937*c*. Jour. Exper. Zoöl., **76**:407.
———. 1937*d*. Biol. Bull., **73**:134.
ALLEN, B. M. 1916. Science, N.S., **44**:1143.
ATWELL, W. J. 1919. Science, **49**:48.
BACQ, Z. M., and FLORKIN, M. 1935. Arch. internat. de physiol., **40**:422.
BÖTTGER, G. 1934. Zeitschr. f. vergl. Physiol., **21**:414.
———. 1936. Klin. Wchnschr., **15**:73.
BUTCHER, E. O. 1930. Jour. Exper. Zoöl., **57**:1.
CARLSON, S. PH. 1935. Proc. Nat. Acad. Sci., **21**:549.
———. 1936. Kungl. Fysiogr. Säll., **6**:1.
DESMOND, W. F. 1924. Anat. Rec., **29**:103.
DIETEL, F. G. 1932. Klin. Wchnschr., **1**:2075.
DOWNES, H. R., and RICHARDS, L. 1935. Jour. Biol. Chem., **110**:81.
FISHER, C. 1936. Endocrinology, **21**:19.
FRASER, A. M. 1937. Jour. Pharmacol. and Exper. Therap., **60**:82.
FRISCH, K. VON. 1911. Arch. ges. Physiol., **138**:319.
GIERSBERG, H. 1932. Zeitschr. f. vergl. Physiol., **18**:369.
HOGBEN, L. T. 1924. The pigmentary effector system. Edinburgh: Oliver & Boyd.
———. 1936. Proc. Roy. Soc. London, B, **120**:142.
HOGBEN, L. T., and GORDON, C. 1930. Jour. Exper. Biol., **7**:286.
HOGBEN, L. T., and SLOME, D. 1931. Proc. Roy. Soc. London, B, **108**:10.
———. 1936. *Ibid.*, **120**:158.
HOGBEN, L. T., and WINTON, F. R. 1922. Biochem. Jour., **16**:619.
HOGG, B. M. 1937. Proc. Soc. Exper. Biol. and Med., **35**:616.
JORES, A. 1932. Klin. Wchnschr., **2**:2116.
———. 1934. *Ibid.*, p. 1269.
JORES, A., and LENSSEN, E. W. 1933. Endokrinologie, **12**:90.
KEEBLE, F., and GAMBLE, F. W. 1904. Phil. Trans. Roy. Soc. London, B, **196**:295.
KLEINHOLZ, L. H. 1935. Biol. Bull., **69**:379.
———. 1936. Proc. Nat. Acad. Sci., **22**:454.
———. 1937. Biol. Bull., **72**:24.
KOLLER, G. 1928. Zeitschr. f. vergl. Physiol., **8**:601.
———. 1930. *Ibid.*, **12**:632.
KOLLER, G., and MEYER, E. 1930. Biol. Zentralbl., **50**:759.
KROGH, A. 1922. The anatomy and physiology of the capillaries. New Haven: Yale University Press.
KROPP, B. 1932. Proc. Nat. Acad. Sci., **18**:690.
KROPP, B., and PERKINS, E. B. 1933. Biol. Bull., **64**:226.
LUNDSTROM, H. M., and BARD, P. 1932. Biol. Bull., **62**:1.
MATTHEWS, S. A. 1933. Biol. Bull., **64**:315.
MEGAŠUR, F. 1912. Arch. f. Entw 'mech., **33**:462.
MEYER, E. 1931. Zool. Jahrb. Abt. allg. Zool. Physiol., **49**:231.
NOBLE, G. K., and BRADLEY, H. T. 1933. Biol. Bull., **64**:289.
PARKER, G. H. 1934. Jour. Exper. Zoöl., **69**:199.
———. 1936. Color changes in animals in relation to nervous activity. Philadelphia: University of Pennsylvania Press.
PARKER, G. H.; BROWN, F. A.; and ODIORNE, J. M. 1935. Proc. Amer. Acad. Arts and Sci., **69**:439.
PERKINS, E. B. 1928. Jour. Exper. Zoöl., **50**:71.
PERKINS, E. B., and KROPP, B. 1932*a*. Mt. Desert Island Biol. Lab. Rept., No. 4.
———. 1932*b*. Biol. Bull., **63**:108.
POUCHET, G. 1876. Jour. anat. et physiol., **12**:1, 113.
RODEWALD, W. 1935. Zeitschr. f. vergl. Physiol., **21**:767.
SAND, A. 1935. Biol. Rev., **10**:361.
SAPHIR, W. 1934. Proc. Soc. Exper. Biol. and Med., **31**:864.
SMITH, P. E. 1916. Science, N.S., **44**:280.

SMITH, P. E., and GRAESER, J. B. 1924. Anat. Rec., **27**:187.
STEHLE, R. L. 1936. Jour. Pharmacol. and Exper. Therap., **57**:1.
SULZBERGER, M. B. 1933. Jour. Amer. Med. Assoc., **100**:1928.
SWINGLE, W. W. 1921. Jour. Exper. Zoöl., **34**:119.
TRENDELENBURG, P. 1929. Die Hormone, Book I. Berlin: J. Springer.
TURNER, H. H. 1935. Endocrinology, **19**:275.
WYKES, U. 1936. Jour. Exper. Biol., **13**:460.
WYMAN, L. C. 1924. Jour. Exper. Zoöl., **39**:73.
YOUNG, A. M. 1934. Amer. Jour. Clin. Path., **4**:485.
YOUNG, J. Z. 1935. Jour. Exper. Biol., **12**:254.
ZONDEK, B. 1935*a*. Glandular physiology and therapy, chap. x. Chicago: American Medical Association.
———. 1935*b*. Jour. Amer. Med. Assoc., **104**:637.
ZONDEK, B., and KROHN, H. 1932*a*. Naturwissenschaften, **20**:134.
———. 1932*b*. Klin. Wchnschr., **10**:405.
———. 1932*c*. *Ibid.*, **11**:849.
———. 1932*d*. *Ibid.*, p. 1293.

PLATE I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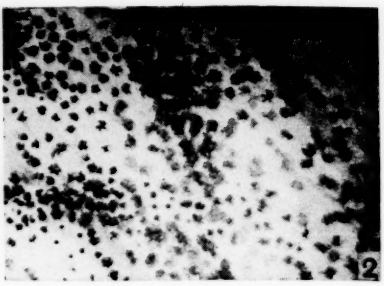
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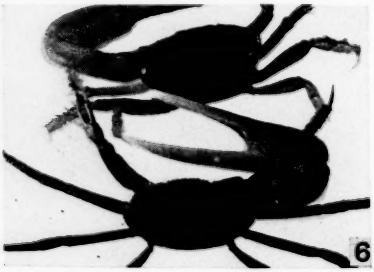
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PLATE I

FIG. 1.—Melanophores of a hypophysectomized tadpole. ×38.

FIG. 2.—Melanophores of a hypophysectomized tadpole after injection of eyestalk hormone. ×38.

FIG. 3—*Left*, hypophysectomized frog injected with crustacean hormone; *center*, hypophysectomized frog injected with intermedin; *right*, hypophysectomized frog uninjected. × ⅓

FIG. 4.—*Left*, hypophysectomized *Anolis* injected with crustacean hormone; *right*, hypophysectomized *Anolis* uninjected. ×½.

FIG. 5.—*Top*, dark coloration produced in blinded crab (*U. pugilator*) injected with intermedin; *bottom*, pale blinded crab, uninjected. ×1.

FIG. 6.—*Top*, *U. pugnax*, blinded, uninjected; *bottom*, *U. pugnax*, normal dark phase. ×½.

ON THE MOTOR AND INHIBITORY INNERVATION OF THE LEGS OF BRACHYURAN DECAPODS

(Two figures)

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VAN HARREVELD and Wiersma (1937) have described the innervation of the muscles of the cheliped of the crayfish *Cambarus clarkii* and *Astacus trowbridgii*, giving a description of the number, the course, and the function of the exciting and inhibiting axons going to the distal six muscles. They had previously found, for the same animals (1936), that the adductor of the claw is innervated by two motor fibers; stimulation of the thicker of these fibers produces a "fast" contraction, while stimulation of the thinner gives rise to a "slow" contraction. It was found that this double motor innervations occurs generally in other muscles of the legs of these animals as well as in those of all marine decapods investigated on this point (Wiersma and van Harreveld, 1938). Indeed, it was concluded, in the paper first mentioned above, that the muscles of the crayfish limb are commonly innervated by two motor axons and an inhibiting one (triple innervation).

In continuation of the foregoing studies the innervation of the legs of several brachyurans has been physiologically investigated. It will be shown that, although the triple innervation is also the rule here, there are, as compared to the innervation of the crayfish, some important differences both in the course and function of the inhibiting fibers (see the scheme shown in Figure 2). Those differences should be taken into account in investigations of the peripheral nerve muscle system of crabs.

METHOD

After removing a cheliped or a walking leg from the animal, the meropodite was opened and the nerve running through it was exposed by removal of the flexor and

Subtribe	Superfamily	Family	Species Used
Oxystomata		Leucosiidae	*Randallia ornata* (Randall)
Dromiacea			No species obtainable
Brachygnatha	Oxyrhyncha	Parthenopidae	*Heterocrypta occidentalis* (Dana)
		Inachidae	*Loxorhynchus grandis* Stimpson
	Brachyrhyncha	Cancridae	*Cancer anthonyi* Rathbun
		Grapsidae	*Pachygrapsus crassipes* Randall

extensor muscles contained in the meropodite. Using the same technique as described in previous papers, the exciting and inhibiting axons were separated and tested by means of two pairs of micromanipulated electrodes, the nerve being submerged either

in sea water or in Pantin's marine solution when not being stimulated. In some cases the contractions were registered, but usually they were only visually observed. Whenever possible, measurements of the diameters of the fibers were made.

Five species of crabs were studied. Their place in the tribe Brachyura is indicated in the classification on the preceding page (after Borradaile).

EXPERIMENTS ON THE ADDUCTOR AND FLEXOR

When the whole leg nerve, prepared as indicated, was placed on the electrodes and faradically stimulated, often no contraction of any muscle was observed upon a stimulation strength which should have been adequate. Very strong stimulation, however, would cause closing of the claw and flexing of the propodite. Sometimes a very narrow range of weak stimuli would produce a contraction, while somewhat stronger stimuli would give no effect. After the nerve had been divided into several bundles, closing of the claw could usually be obtained from one of these. Further division of this bundle always yielded two fibers which were responsible for this contraction. In agreement with the results found by Wiersma and van Harreveld (1938), stimulation of the thinner fiber at frequencies below 50 per second usually produced a contraction which was more rapid than that resulting upon stimulation of the thicker fiber under the same conditions. As the frequency was raised above 50 per second, a transition frequency was found at which the two types of contraction were quite similar in appearance; at still higher frequencies, the adduction elicited by means of the thicker axon was always faster than that caused by the thinner one. The transition frequency was rather inconstant in the present experiments, varying not only with the species but also with the state of fatigue of the preparation. Since it is likely that the quick fatigue is a feature of the summer season, a more detailed comparative study of the transition frequency was not made at this time.

Upon simultaneous stimulation with one pair of electrodes of all the fiber bundles remaining after isolations of the two adductor motor axons, one or both of the latter axons being also stimulated with the other pair of electrodes, it was found that usually the contraction of the adductor was either suppressed entirely or reduced considerably. Next, the bundle responsible for this inhibition was isolated and divided. When the inhibiting axon was found, excitation of the remaining fibers of this bundle produced flexion of the propodite, an effect which had not been visible before. Further isolation yielded two fibers of different diameters which caused this contraction, indicating the existence of a double motor innervation here. These two motor fibers usually run quite close to the inhibiting axon mentioned above. When this inhibiting fiber was stimulated simultaneously with one or both of the flexor motor fibers, it was found that flexion inhibition also occurred; in other words, the adductor and the flexor have one inhibitory fiber in common. This fiber, then, is able to suppress or reduce the contractions produced by excitation of any one of four motor fibers.

In the large walking legs of *Loxorhynchus* it was possible to record the contractions of the adductor and of the flexor separately, the tendon of the latter muscle being connected directly to a lever, while the contraction of the adductor was recorded with a lever connected to the dactylopodite. Figure 1 shows the effect of stimulation of the inhibitor axon upon the contractions produced by the stimulation of the four motor fibers simultaneously. However, such records were difficult to obtain because the neces-

sary stimulation of the inhibitor fiber during its preparation often resulted in fatigue of inhibition before isolation of the inhibitor had been accomplished.

EXPERIMENTS ON THE ABDUCTOR AND EXTENSOR

In the crayfish the flexor of the propodite has its own inhibitory axon, while the slow contraction of the adductor is inhibited, though to a slight extent, by the same fiber which inhibits the contraction of the extensor of the propodite (Hoffman, 1914; Serkoff, 1936; Marmont and Wiersma, unpublished). In crabs, however, the function of the extensor inhibitor was found to be restricted to the extensor muscle. As in the crayfish, the abductor and the extensor are innervated by a single common motor axon, but preparation of this motor axon and of the extensor inhibitor fiber is more difficult in

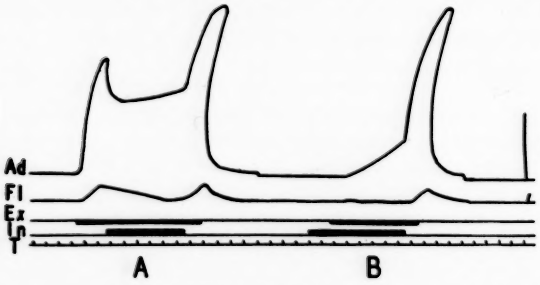

FIG. 1.—Preparation from *Loxorhynchus grandis* showing simultaneous inhibition of the contractions of the adductor and of the flexor of the propodite produced by stimulating their common inhibiting axon. The two motor fibers to the adductor and the two to the flexor are faradically stimulated on one pair of electrodes; the inhibitor is similarly stimulated by another pair. In *A*, inhibition is applied in the midst of a contraction; in *B*, the inhibition is started before excitation. In this preparation adductor inhibition is not complete. *Ad* is the isotonic contraction of the adductor; *Fl*, that of the flexor; *Ex* and *In* show the periods when excitation and inhibition were given, respectively; *T* is the time in seconds.

crabs because the inhibitor fiber of the abductor runs in the same bundle. This means that no contraction of either muscle can be obtained until at least one of these three fibers has been separated from the others. This difficulty is mitigated by the facts that these rather large axons run in a bundle containing only a few small sensory fibers and that the bundle occupies a fairly constant position in the whole nerve. Since the extensor and the abductor muscles have one single motor fiber in common, they possess only one type of contraction and must be considered, as in the crayfish, as parts of one motor unit, being separately inhibitable.

SCHEME OF INNERVATION

In Figure 2 the innervation to the four distal muscles found by this method in all the species of crabs investigated is compared to that found in the crayfish. It will be seen that the number of fibers is the same in both types, not only in total number but also as divided according to the function: "fast," "slow," or inhibitory. However, the following differences are present. In the crabs the opener inhibitor does not run in the bundle containing the adductor motor axons, at least not distally to the beginning of the meropodite. In these animals there is no extension of the extensor inhibitor to the

closer muscle, as in the crayfish (we term such an extension the "X-fiber"), but instead there is an extension of the flexor inhibitor to the closer (Y-fiber). This Y-fiber is much more effective in producing inhibition of the adductor muscle than the X-fiber. It is interesting that in these two suborders of the decapods, whose limbs are morphologically quite similar, inhibition of the adductor should have been taken over in one group by the inhibitor of the flexor of the propodite and in the other group by the inhibitor of the extensor of the propodite

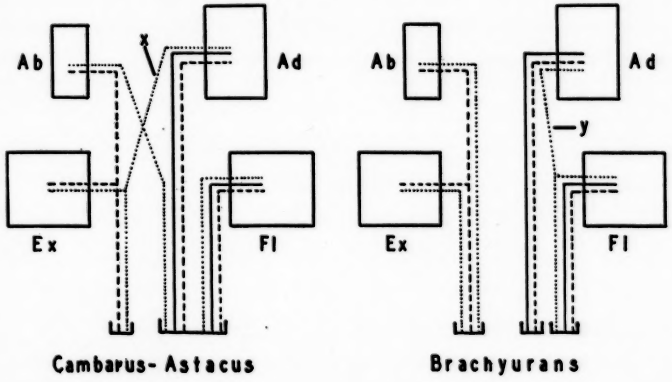

FIG. 2.—Diagrams of the innervation of the distal four muscles of the chelipeds or walking legs of the crayfish and of brachyurans. The unbroken lines represent an axon causing a "fast" contraction; the dashes represent those causing a "slow" contraction; and the dotted lines represent inhibiting axons. *Ab* is the abductor; *Ad*, the adductor; *Ex*, the extensor of the propodite; and *Fl*, the flexor of the propodite. *X* and *Y* mark the "X" and "Y" fibers.

Several anomuran decapods were given a preliminary investigation, namely, *Pagurus ochotensis*, Brandt, *Paguristes bakeri*, Holmes and *Blepharipoda occidentalis*, Randall. Physiologically, their innervation seemed identical with that occurring in the crayfish, except that no evidence was found for the existence of the X-fiber.

FIBER DIAMETERS

Measurements of the diameters of fibers from preparations of *Loxorhynchus*, *Cancer*, and *Pachygrapsus* are given in Table 1. As in the crayfish, the motor and the inhibitor axons are of large size, compared to the sensory axons. The diameter of any specific fiber bears no relation to the size of the species: the largest fibers occur in the walking legs of *Pachygrapsus*, which is one of the smaller species investigated. *Cancer* and *Loxorhynchus*, the largest species, had fibers of a diameter corresponding to those of the much smaller crayfish.

SUMMARY

The innervation of the legs of five species of brachyuran decapod crustaceans was investigated by isolating the motor and inhibiting axons going to the distal four muscles. The function of those fibers was observed upon stimulation.

As in the crayfish, the abductor and the extensor of the propodite muscles were found to be parts of one motor unit, but each possesses an inhibitor fiber; these three fibers run in the same bundle in the meropodite. The adductor and the flexor of the propodite each has its own "fast" and "slow" axons; however, only one inhibitory fiber is present, and this inhibitor is able to suppress or decrease contractions of both muscles.

TABLE 1

FIBER DIAMETERS

Species	Adductor		Common Inhibitor	Flexor		Abductor Extensor	Abductor Inhibitor	Extensor Inhibitor
	Fast	Slow		Fast	Slow			
Loxorhynchus:								
Claw	30 μ	24 μ	21 μ	36 μ	27 μ	.....	.....	.....
	42	30	.....	.....	.....	.....	.....	.....
	54	42	.....	.....	.....	.....	.....	.....
Leg	48	46	.....	.....	.....	.....	.....	.....
Cancer:								
Leg	45	36	.....	.....	.....	42 μ	33 μ	36 μ
Pachygrapsus:								
Claw	57	48	27	54	48	60	42	45
	60	42	.....	.....	.....	54	45	42
	84	51	.....	.....	.....	60	45	45
Leg	84	54	.....	.....	.....	.....	.....	.....
	66	42	36	66	40	.....	.....	.....
	.....	.....	.....	60	.....	45	42	42

LITERATURE CITED

HARREVELD, A. VAN, and WIERSMA, C. A. G. 1936. The double motor innervation of the adductor muscle in the claw of the crayfish. Jour. Physiol., **88**:78.

———. 1937. The triple innervation of crayfish muscle and its function in contraction and inhibition. Jour. Exper. Biol. **14**:448.

HOFFMAN, P., 1914. Über die doppelte Innervation der Krebsmuskeln. Zugleich ein Beitrag zur Kenntnis nervöser Hemmungen. Zeitschr. f. Biol., **63**:411.

SERKOFF, PH. N. 1936. Elektrophysiologische Untersuchung der peripheren Hemmung im Scherenschlieszer des Fluszkrebses. Pflügers Arch., **237**:631.

WIERSMA, C. A. G., and HARREVELD, A. VAN. 1938. A comparative study of the double motor innervation in marine crustaceans. Jour. Exper. Biol. **15**:18.

THE OLFACTORY REACTIONS OF DROSOPHILA MELANOGASTER MEIGEN TO THE PRODUCTS OF FERMENTING BANANA[1]

(Three Figures)

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Department of Zoölogy, University of Minnesota

THE most extensive early work on the olfactory sense of *Drosophila melanogaster* was that of Barrows (1907). In the course of his work Barrows made some preliminary experiments to determine substances which called forth positive reactions. These experiments consisted of setting up a trap, containing the odorous substance to be tested, in a glass cylinder closed by a glass plate. The air in the cylinder was kept moist by the evaporation of distilled water. A hundred flies which had been previously starved for 24 hours were put into the cylinder, and at the end of another 24 hours the number caught in the trap was recorded. The trap used by Barrows consisted of a vial through the cork of which extended a piece of glass tubing (Fig. 1*b*). Following these preliminary experiments, Barrows used an olfactometer, in which the flies, while passing from a vessel in response to light, were given a "choice" of entering a trap on one side containing odor or a trap on the other side containing water.

Barrows (p. 536) sums up his results as follows:

> These flies are positively chemotropic to amyl and especially ethyl alcohol, acetic and lactic acids and acetic ether.
>
> Acetic ether, isobutyl acetate and methyl acetate, when added in small amounts to 10 per cent ethyl alcohol, greatly increase its attractiveness. A similar increase is noted where acetic or butyric acids are added to the alcohol. All these organic substances are found in fermenting fruits.
>
> The optimum strengths of ethyl alcohol and acetic acid as determined by the number of positive reactions given to different strengths is 20 and 5 per cent, respectively, while a mixture containing $2\frac{1}{2}$ per cent alcohol and $\frac{5}{8}$ per cent acetic acid gives a slightly higher number of positive reactions than is given by either 5 per cent acetic acid or 20 per cent ethyl alcohol.

The possibility of improving the methods used in Barrows' preliminary experiments and of more accurately measuring the reactions of *Drosophila* to the products of fermenting banana led to the present experiments.

METHODS

Effect of trap form on catch.—The best trap is obviously one which will catch and retain the greatest number of flies. Trap form is thus a matter of prime importance. Various

[1] The author wishes to express her appreciation to Dr. D. E. Minnich, her adviser, not only for the suggestion of the problem but also for his invaluable criticism and his inspiring enthusiasm; to acknowledge her gratitude to Dr. C. P. Oliver, who taught her how to culture *D. melanogaster;* to Dr. A. C. Hodson, who has given her some helpful suggestions; and to Dr. Joseph Valasek, who measured the range of the red lamp which was used. She wishes to thank Dr. R. N. Danielson and Dr. Elizabeth Brown Chase for valuable suggestions and German translations, and she wishes also to express her indebtedness to other students who have worked on this problem, among whom are Gertrude Fink McIntosh, Edith Quamme Meineke, Philip Le Compte, and Patty Hynes Wallace.

types of traps tried by previous workers in this laboratory are illustrated in Figure 1. No one of these proved satisfactory. The form of trap finally chosen may be seen in the experimental setup (Fig. 2). It is made from a piece of glass tubing $8\frac{1}{2}$ cm. long and 28 mm. in internal diameter, one end of which has been drawn out and bent in to form an

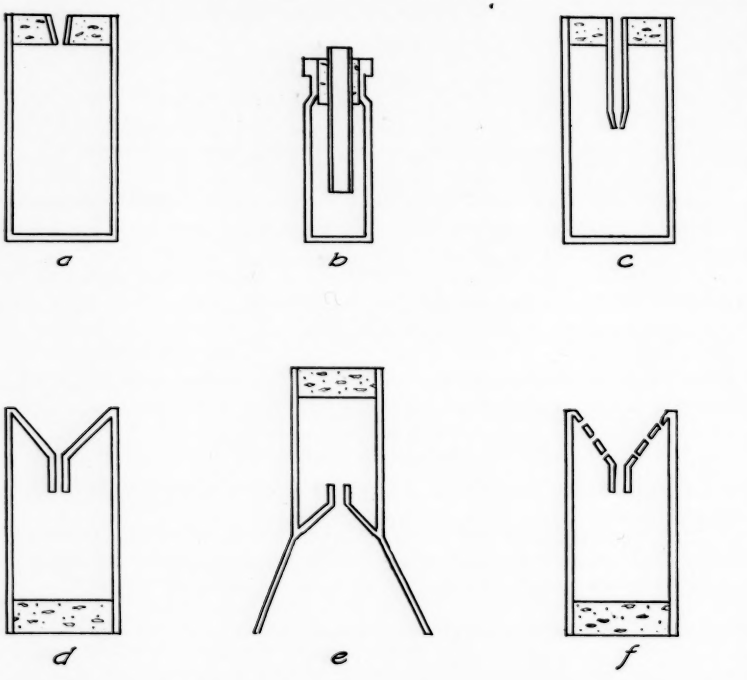

FIG. 1.—Traps used by other workers

inverted funnel-shaped opening. The lower end of the funnel opening is 2.1 mm. in diameter. The other end of the tube is closed by a cork. In setting up the trap, the odor solution is put into a small glass dish or thimble and set on the cork, and the trap is placed over it.

With a bait of fermenting banana the foregoing trap caught about 90 flies out of 100 in 12 hours, other pertinent factors being under control. A number of such traps were made, and an identification mark scratched on each. In eight experiments 7,113 flies were caught out of 7,755, or 91.7 per cent. Nine out of the eleven traps used caught

over 90 per cent, while the two remaining traps caught 87.9 per cent each. The variation in catch ranged from 90 to 98 per cent in the best trap and from 65 to 99 per cent in the poorest trap. The high percentage of flies caught and retained in the traps over a period of 12 hours signified that the escape was small.

The greatest difficulty in the selection of a trap was the variability in catch irrespective of the type of trap employed. This variability led to the investigation of other factors which might be important in influencing the number of flies caught.

Effect of other factors on catch.—Temperature is important for several reasons. The rate at which the fly moves decreases as temperature decreases. Fewer flies are, there-

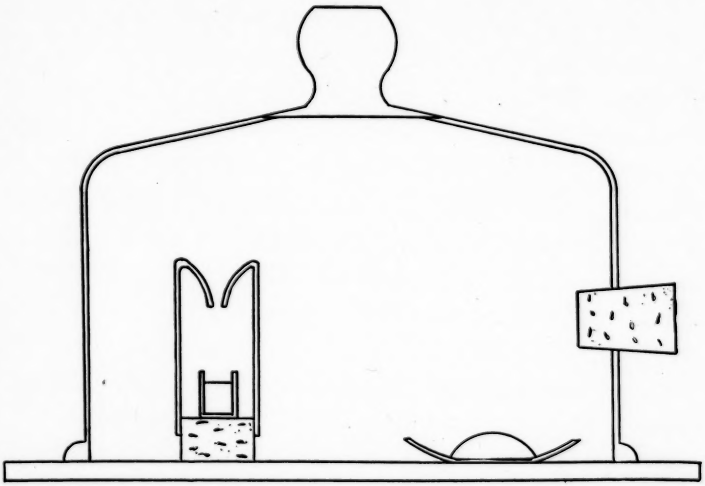

FIG. 2.—The experimental setup, consisting of a bell jar on a sand-blasted glass plate, containing a trap with the odor solution and a watch glass of cotton soaked with distilled water to regulate humidity. The hole in the side of the bell jar is used at the beginning of the experiment to insert the counter and at the end of the experiment for etherization of the flies.

fore, caught in the traps at lower temperatures. The fly may make some internal physiological adjustment to the temperature of its environment, so that it may make a difference if the fly is experimented upon at the same temperature at which it is cultured or at other temperatures. This is a question for experimentation.

The temperature at which flies are cultured is also important in determining the size of the flies; and when the flies are small, the number caught is less than when they are large. Much recent work has been done on this question of relation of temperature to size. Alpatov (1929) found that *Drosophila* reared at 18° C. was larger in a series of bodily dimensions than when reared at 28° C. His method of determining size consisted of taking nine measurements on different parts of each individual. Hersh (1924) and Baron (1935) have studied the effects of temperature on the facet number in *D. melanogaster;* and Stanley (1935) has studied the effect of temperature on wing length, with similar results.

The writer used the average weight per hundred to determine the relative size of flies raised at different temperatures. Since the flies rapidly lost weight because of the evaporation of water from their bodies after etherization, it was necessary to weigh them before counting them. With flies weighing 113.2 mg. per hundred, 90.4 per cent of 1,007 were caught; while with flies weighing 87.7 mg. per hundred, only 62 per cent of 1,008 were caught.

There are three possible reasons why fewer small flies are caught: first, the smaller fly, after entering the trap, may escape more easily than the larger fly; second, the smaller fly may be less active, so that fewer enter the trap; and third, the smaller fly may be less responsive to odors. To test these possibilities the following experiments were performed.

To test the possibility of escape, a hundred flies, weighing about 41.1 mg. per hundred, were counted into each of three traps. Each trap was set under a bell jar, and a dish of fermenting banana was also set under each bell jar. At the end of 2 hours it was found that an average of 36.3 per cent of the small flies had escaped, while an average of 6.2 per cent of the large had escaped. This shows that a considerable number of flies do escape and that it is easier for the small flies to escape than for those of larger size.

In a second experiment ten traps each containing 100 flies (which had been raised at 21° C.) were set as in the previous experiment. After 12 hours it was found that the average percentage of escape for the males was 5.6, while that for the females was 2.4. Since females in *Drosophila* are larger than males, this difference may be due to size.

These experiments thus show that the flies can and do escape when there is an attractive odor outside the trap. The percentage of escape, however, is small when large flies are used.

The effect of activity on catch was tested as follows. Since the visible spectrum of some of the insects is shifted toward the ultra-violet, it seemed possible to find a red light by which the experimenter could observe the actions of the flies under conditions which approach darkness for the flies.[2] The wattage of the lamp chosen was 25; and its range was from 617 $\mu\mu$ to 758 $\mu\mu$, with the maximum brightness at 664 $\mu\mu$. According to Bertholf (1932), the extent of the spectrum for *D. melanogaster* is from 235 $\mu\mu$ to about 650 $\mu\mu$. The stimulating efficiency, however, of the longer wave-lengths is low, the relative stimulating efficiency at 600 $\mu\mu$ being 0.7; at 625 $\mu\mu$, 0.1; and beyond 625 $\mu\mu$ unmeasured.

The red electric light was put into a box (8 × 6 × 4 inches) which was open at the top. Upon the box was placed a sanded glass plate upon which sat a bell jar containing 200 flies. After the flies had been starved for 10 hours, the trap containing the odor was slipped up under the bell jar and all the lights were turned out except the red light. The entrance of the flies into the trap was observed for 15 minutes. During this time no flies were observed to escape from the trap in any of the trials. The factor of escape, therefore, did not enter into these experiments. At the end of the 15 minutes the flies were etherized and counted, and the percentages of males and females were recorded.

In the first set of trials no bait was used in the traps. The flies were divided into four groups of 200 each, weighing 40 mg., 74.3 mg., 112.8 mg., and 117 mg. per hundred, respectively. Each group was placed under a bell jar. More large flies were caught than small flies, and more females than males. The percentage of females caught in each of

[2] During the time of trial the flies were kept in darkness.

ERRATUM

In the third paragraph of XI, No. 3 (July), 320, the first sentence should read: "To test the possibility of escape, a hundred flies, weighing about 41.1 mg. per hundred, were counted into each of three traps, and a hundred large flies, weighing about 124.1 mg. per hundred, were counted into each of three other traps."

the four groups was 6.4, 16.0, 32.4, and 36.4, respectively; and the corresponding percentage of males caught was 1.1, 5.6, 16.6, and 20.6.

In the second set of trials the flies were all of the same weight, 117 mg. per hundred. The following baits were used: fermenting banana, 10 per cent acetic acid, 15 per cent ethyl alcohol, and 0.01 per cent amyl acetate. The fermenting banana caught 28.0 per cent females and 4.6 per cent males, the acetic acid caught 0 per cent females and 1 per cent males, the ethyl alcohol caught 34.9 per cent females and 10.6 per cent males, and the amyl acetate caught 26.2 per cent females and 14.2 per cent males.

It is interesting to note that, when a *Drosophila* entered an empty trap, it crept right in without hesitating at the opening; but when it entered an odor trap, it hesitated at this point. With the 10 per cent acetic acid, which acted decidedly as a repellent, the flies, on reaching the opening, either backed out or turned and crept out. The one male which entered flew in directly from the air.

Since no flies escaped, the red-light experiments with empty traps clearly show that there is a difference in activity. The small, undernourished flies raised at high temperatures in crowded conditions are less energetic and active than larger flies raised under optimum conditions. The fact that more females were caught than males must, at least in part, be due to a greater activity on the part of the female or, perhaps better, to a difference in the kind of activity in the males and females. Since females are searching for a place to lay their eggs, they may enter traps oftener than males.

The third point raised above, viz., the relation between the size of the fly and the response to odor, was not determined.

Elimination of anesthetic before trials.—Barrows (1907, p. 531) states: "It should be noted that the process of etherizing has no lasting effect on the ability of the flies to scent food, i.e., normal flies after recovery from ether find food with as great certainty as they did before etherization." He says nothing, however, about the intensity of the etherization or about the time necessary for recovery. The writer has found that under certain conditions etherization has an effect upon the olfactory sense for at least 12 hours after etherization, and perhaps the injury may be permanent.

It was decided, therefore, to use only unetherized flies,[3] which necessitated a special technique. To count the flies without etherizing them, a counter, similar to the one Pearl (1928) used for population counts, was made. The counter (Fig. 3) is made from a piece of glass tubing about 24 cm. long and with an internal diameter of 21 mm. One end is drawn to a funnel opening, 1.8 mm. in diameter and just large enough to let one fly go through at a time; while the other end is flared to fit the top

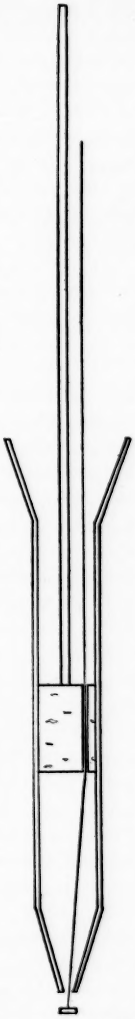

FIG. 3.—The counter used to count flies without etherization.

[3] The etherizer consisted of a half-pint milk bottle into the top of which was put an aluminum funnel. The upper edge of the funnel was bent to fit around the top of the bottle; and the lower edge of the funnel tube was slit up and bent back to hold some cotton, which was soaked with ether each time the etherizer was used.

of a pint milk bottle. The plunger, which can be drawn up and down in the tube, is made of cork with a brass rod for a handle. The little valve which closes the opening is a cardboard washer to which is glued a long piece of piano wire. The wire extends from the valve up through the tube and through a small hole burned through the cork to about 4 cm. beyond the tube. By means of this wire the valve may be opened or closed to regulate the number of flies which go through. As a result of using unetherized flies, larger and more constant catches were obtained.

Cultures.—The standard procedure was followed in making the yeast food. The following proportions were used for 20 bottles:

250 cc. bananas	10 gm. agar
125 cc. white Karo syrup	½ cc. 10 per cent formalin
625 cc. water	10 yeast cakes dissolved in water
100 cc. water (added after the agar solution boils)	

Twenty flies were counted into each culture bottle (pint milk bottle) without etherizing, since etherizing retards the laying of eggs and the bottles are more apt to become moldy. Most of the flies used in these experiments were raised at 20° C. Flies raised on yeast food and at this temperature are of good size, weighing about 117 mg. per hundred.

General procedure.—The experiments were carried on and the cultures were developed in a small inside room which was about 11 feet long, 8 feet wide, and 11 feet high, maintained at a constant temperature of 20° C. The glassware was washed, the cultures were set, and the odor solutions were made in a near-by room.

The glassware was washed in tap water, rinsed in cleaning solution, tap water, and distilled water, and air dried. The small pieces and the milk bottles were dried in an electric oven, and the bell jars and the glass plates were dried with an electric fan.

For steady experimentation a culture of 20 bottles was set every other day. When the young began to pupate, the parent flies were removed from the culture bottles. Three days after hatching started, the flies were emptied from culture bottles into 2 food bottles by means of an aluminum funnel. The food bottles were quart milk bottles into which about 4 tablespoonfuls of yeast food had set. A half paper towel was put into each bottle to take care of the moisture, and the top was closed with a plug of cotton. The following day some of the flies were emptied into the counter, to be used in experiments. These flies were from 1 to 4 days old. On the second day flies from the same food bottles were used. These were from 2 to 5 days old. No significant difference in reaction was found between the 1–4-day flies and the 2–5-day flies.

In setting up an experiment, 12 bell jars were set on sand-blasted glass plates (Fig. 2) along the edge of a table in the constant-temperature room. A watch glass containing a piece of cotton soaked in distilled water was put under each bell jar, and a fresh cork was put into the hole on the side. A hundred flies were then counted into each bell jar, as previously described; the light was turned off; and the flies were left without food but with water for 10 hours.[4] At the end of this time the light was turned on, and an odor trap was slipped up under each bell jar by pulling the bell jar a little over the edge of the table. The flies were again left in the dark, this time for 12 hours,[4] after which they were etherized by pushing a plug of ether-soaked cotton through the hole in the side of the bell jar and replacing the cork. A count was then made, and the percentage of females

[4] The period used by Barrows was 24 hours.

and males caught was recorded. The constant-temperature room was then made ready for the next experiment by the removal of the glassware and by airing.

It is well known that *D. melanogaster* is positively phototropic to a high degree. This factor was made use of in these experiments, not only to attract the flies to the top of the bell jar while the trap was being put in, but also to attract the flies through the opening of the counter. The end of the counter was put into a hole which had been bored into the side of the bell jar, and was turned toward the light. The rate at which the flies moved appeared to depend upon the intensity of the light and also upon other factors, such as the amount of moisture in the tube of the counter (moisture collects upon the sides of the tube of the counter if the flies are left in it for any length of time) and also upon the state of general excitement of the flies. This state could be brought about by emptying the flies from the food bottles into the counter. In regard to this state of general excitement, McEwen (1918) says that this fly is not phototropic unless it is in a certain physiological state brought on by, or at least accompanied by, activity.

EXPERIMENTS

It is well known that *Drosophila* is strongly positive to fermenting banana. The question naturally arises as to just which of the products of fermentation calls forth a positive response and in what proportions. Von Loesecke (1929) has studied such fermentations, and the following data are taken from his work. Banana mash contains 16 per cent fermentable sugars. With fermentation the ethyl alcohol content varies from 6.55 to 10.12 per cent. Acetic fermentation gives 5.72 per cent acetic acid. The reactions of *Drosophila* to ethyl alcohol solutions varying from 5 to 30 per cent and to acetic acid solutions varying from 0.1 to 5 per cent were accordingly studied.[5]

Ethyl alcohol.—Ten experiments were made, in each of which 100 flies were tested with each concentration of alcohol and with each of three controls, viz. an empty trap, a trap containing distilled water, and a trap containing fermenting banana.

The results of the experiments are given in Table 1. It is interesting to note that more flies were caught in empty traps than in water traps. Distilled water alone, therefore, acted as a repellent under the conditions of the experiment.

The positive response of females, as shown by the number of individuals responding, increased with an increase of concentration up to between 10 and 15 per cent, beyond which point the positive response decreased with an increase in concentration. Of the solutions tried, the lowest, a 5 per cent solution, called forth a greater positive response among the males than any other solution. With an increase in concentration the positive response of the males decreased. A definite negative response was shown by both males and females to solutions above 25 per cent, since the percentage caught with solutions above this concentration was less than with empty or water traps.

The most plausible interpretation of the difference in response between males and females is that it is a difference in response to odors and not entirely a difference in activity. If the difference in the reactions of males and females to any bait were due entirely to a difference in activity, we would expect this difference to remain constant, irrespective of the bait used. That it does not remain constant shows that there is a difference in reaction to the odor of the bait.

Experiments with acetic acid.—The methods used in the study of the reactions of

[5] Solutions were made by volume.

Drosophila to acetic acid were the same as those used with ethyl alcohol solutions. The results of eight experiments, using 800 flies for each concentration, are given in Table 2. Solutions of 0.1, 0.2, 0.4, 0.6, 1, and 5 per cent concentrations were used. The writer's results differ greatly from those of Barrows (1907). He found the optimum strength

TABLE 1

SUMMARY OF TEN EXPERIMENTS, SHOWING CONTROLS AND RESPONSE TO ETHYL ALCOHOL

Period of starvation, 10 hours. Time of trial, 12 hours. Age of flies, 1–4 and 2–5 days. Temperature, 20° C.

Bait	No. of Flies Caught in Trap		No. of Flies Not Caught		Percentage Caught	
	Female	Male	Female	Male	Female	Male
Empty	107	149	252	506	29.8	22.7
Water	51	65	296	594	14.7	9.9
Banana	444	379	24	157	94.9	70.7
5 per cent alcohol	274	211	189	321	59.2	39.7
10 per cent alcohol	336	163	185	324	64.5	33.5
15 per cent alcohol	259	199	146	380	63.9	34.4
20 per cent alcohol	239	72	353	355	40.4	16.9
25 per cent alcohol	89	32	434	437	13.2	6.8
30 per cent alcohol	34	17	454	510	7.0	3.2

TABLE 2

SUMMARY OF EIGHT EXPERIMENTS, SHOWING CONTROLS AND RESPONSE TO ACETIC ACID

Period of starvation, 10 hours. Time of trial, 12 hours. Age of flies, 1–4 and 2–5 days. Temperature, 20° C.

Bait	No. of Flies Caught in Trap		No. of Flies Not Caught		Percentage Caught	
	Females	Males	Females	Males	Females	Males
Water	54	81	233	441	18.8	15.5
Banana	252	436	8	116	96.9	78.9
0.1 per cent acid	151	64	240	334	38.6	16.1
0.2 per cent acid	196	159	220	229	47.1	40.9
0.4 per cent acid	279	97	193	229	59.1	29.7
0.6 per cent acid	236	107	198	281	54.4	27.6
1 per cent acid	115	83	316	288	26.7	22.4
5 per cent acid	16	3	396	396	3.9	.8

of acetic acid, as determined by the number of positive reactions given to different strengths, to be 5 per cent (including the responses of both males and females). The writer found 0.4 per cent to be optimum for females and 0.2 per cent to be optimum for males, while a solution of 5 per cent or greater (tried in preliminary experiments) acted as a repellent. A 0.4 per cent solution caught 59.1 per cent of females, and a 0.2 per cent solution caught 40.9 per cent of males. A 5 per cent solution caught 3.9 per cent females and 0.8 per cent males.

In regard to the repellent action of acetic acid, it was observed during the experiment with the red light that with a 10 per cent solution as bait the flies crawled down the funnel of the trap to the opening and then either backed out or turned and crawled out. Some stopped part way up the funnel to clean the head and abdomen.

SUMMARY

A series of experiments to determine the olfactory reactions of *D. melanogaster* to solutions of various concentrations of ethyl alcohol and acetic acid is reported, with the following results.

1. A trap has been perfected which will catch about 90 flies out of 100 in 12 hours when fermenting banana is used as bait.

2. A new valve has been invented for Pearl's counter which makes it possible to count out a definite number of flies at a time. This makes it unnecessary to etherize flies in order to count them, and thus does away with the possibility of the detrimental effects of etherization upon the olfactory sense.

3. A method of observing the activity of *Drosophila* under conditions which approach darkness for the fly has been described.

4. Large flies are more active than small flies.

5. Females are more active than males, as indicated by the fact that more females are caught in empty traps than males.

6. There is a difference between males and females in their response to odors.

7. Excessive moisture acts as a repellent, as is shown by the fact that empty traps catch more flies than water traps.

8. *Drosophila* females are strongly positive to solutions of ethyl alcohol up to 25 per cent, with a maximum response at 10–15 per cent; and *Drosophila* males are definitely positive to ethyl alcohol solutions up to 15 per cent, with a maximum response at or below 5 per cent.

9. *Drosophila* females are strongly positive to acetic acid in concentrations up to 1 per cent, with a maximum response at 0.4 per cent; and *Drosophila* males are positive to acetic acid in concentrations up to 1 per cent, with a maximum response at 0.2 per cent.

10. Solutions of ethyl alcohol above 25 per cent and of acetic acid above 5 per cent act as repellents to both males and females.

LITERATURE CITED

ALPATOV, W. W. 1929. Influence of temperature on *Drosophila melanogaster*. Amer. Nat., **63**:37.

BARON, ABRAHAM L. 1935. Facet number in *Drosophila melanogaster* as influenced by certain genetic and environmental factors. Jour. Exper. Zoöl., **70**:461.

BARROWS, WILLIAM MORTON. 1907. Reactions of the pomace fly to odorous substances. Jour. Exper. Zoöl., **4**:515.

BERTHOLF, L. M. 1932. The extent of the spectrum for *Drosophila* and the distribution of stimulative efficiency in it. Zeitschr. f. wiss. Biol., Abt. C, Zeitschr. f. vergl. Physiol., **18**:32.

HERSH, A. H. 1924. The effects of temperature upon the heterozygotes in the bar series of *Drosophila*. Jour. Exper. Zoöl., **39**:55.

LOESECKE, HARRY VON. 1929. Preparation of banana vinegar. Ind. and Eng. Chem., **21**:175.

MCEWEN, R. S. 1918. The reactions to light and gravity in *Drosophila* and its mutants. Jour. Exper. Zoöl., **25**:49.

PEARL, RAYMOND. 1928. The rate of living. New York: Knopf.

STANLEY, WILLARD F. 1935. The effects of temperature upon the wing size in *Drosophila*. Jour. Exper. Zoöl., **69**:459.

RACE AND CONJUGATION OF PARAMECIUM

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MANY investigators, among whom the most recent are Sonneborn and Cohen (1936), have reported that some races of *Paramecium* and of other protozoa conjugate readily while other races of the same species conjugate rarely, if at all, under apparently identical conditions. It is possible that different races respond to variations in different environmental factors, for changes in each of the following factors: (1) food supply (Maupas, 1889, and many others), (2) metabolites (Chatton and Chatton, 1931), (3) pH (Austin, 1927), (4) salt type and concentration of the medium (Enriques, 1909, and Zweibaum, 1912), (5) population density (Mast, 1917), (6) oxygen tension (Enriques, 1909), (7) temperature (Prandtl, 1906), and (8) the type of substrate (Baitsell, 1912) in which the organisms are grown, have been invoked but not conclusively proved as possible factors which, singly or concomitantly with other factors, induce conjugation in conjugating races of diverse protozoans.

In a recent study of a conjugating race of *Paramecium multimicronucleatum* Powers and Mitchell, when most of the other factors were kept relatively constant, conjugation was found to be closely correlated with a decrease in available food after a period of plenty (Giese, 1935) when accompanied by a simultaneous change in temperature from 26° to 20° C. The change in temperature alone was ineffective in inducing conjugation. It seemed interesting (1) to compare the conditions necessary for induction of conjugation in a number of other races of the same species of *Paramecium*, and (2) to determine, should any of the other races fail to conjugate under the conditions which are correlated with conjugation in the race already studied, whether changes in any of the other environmental factors listed above might induce conjugation.

MATERIALS AND METHODS

The races of *Paramecium*, which for convenience have been numbered 1, 2, 6, 7, 8, 9, and 11, were all isolated in the vicinity of Stanford University but in different habitats and at widely separated localities. Race 1 was isolated in the fall of 1932; race 11 was obtained from this race in 1935; all the others were isolated in the spring of 1936.

The culture methods were similar in most respects to those already reported. Briefly, the paramecia were grown on a single strain of bacteria (*Pseudomonas ovalis*) in buffered (pH 7.0) lettuce extract at 26 ± 0.1° C., one animal being inoculated per cubic centimeter of medium, and were prevented from conjugation by subculturing until conjugation tests were made ($3\frac{1}{2}$ days after inoculation). The main change in culture method from that previously reported was filtration of the medium, instead of decanting, to eliminate the particles of lettuce which interfered with the conjugation tests. Preliminary tests showed that medium filtered through ordinary qualitative filter paper was unfit for use; thus, whereas in controls there were, on the average, 284 animals per cubic centimeter 3 days following subculture, in filtered medium there were but 5 per cubic centimeter. Medium filtered through Seitz and Chamberlain bacteriological filters was

so toxic that the animals rarely divided, became bloated and burst, dying long before the death of controls starving in balanced inorganic salt solution. It was later found that the qualitative paper could be rendered innocuous by a wash with hot lettuce medium (not, however, by hot distilled water). Furthermore, quantitative paper was generally non-toxic, but filtration was slow and tedious. Coarse, gray filter paper did not seem to introduce any toxic materials and was far more convenient than quantitative paper; therefore, following a preliminary "safety" wash with hot culture fluid, it was used for filtering the culture media employed in these experiments.

The method used for inducing conjugation was essentially that previously described (Giese, 1935). Animals from cultures in which multiplication had practically ceased were concentrated twenty fold by centrifugation and placed in watch glasses at 20° C. While concentration is unnecessary, it results in a more rapid reduction of remaining food. A change in temperature was found to be unnecessary for some of the races; but since the epidemic lasts longer at the lower temperature, such a change facilitates observations. When the effect of a given solution was to be studied, the paramecia were washed and suspended in that solution in the manner previously described.

The isolation culture method used to determine viability of exconjugants of the different races studied was as follows. A single conjugant pair was placed in a tube 5 mm. by 7 cm., containing about $\frac{3}{4}$ cc. of the lettuce medium described, which was diluted half and half with distilled water, a very light inoculation with *P. ovalis* being made at the same time. Observations were then made at 12-hour intervals. Previous studies had shown that higher concentration of culture medium is unfavorable, since multiplication of exconjugants is too slow to keep apace of bacterial development.

Dishes and glassware were carefully cleaned and sterilized by autoclaving or dry sterilization. Bacteriological precautions were observed for all organic solutions and cultures in such solutions; inorganic solutions were sterilized to prevent entrance of extraneous protozoans. Most of the experiments were performed in Columbia watch glasses or in 1-inch Syracuse watch glasses; some in 3-inch watch crystals. All were appropriately moist-chambered.

The inorganic balanced salt solution referred to in the paper is a modified form of the solution used by Barker and Taylor (1931), consisting of 6 per cent solutions of the following salts mixed in the proportions given: NaCl, 1,000 parts; $MgCl_2$, 78 parts; $MgSO_4$, 38 parts; KCl, 22 parts; $CaCl_2$, 10 parts. This mixture was diluted five hundred times and buffered at pH 7.0 with 1 part stock buffer added to 9 parts salt solution. The stock buffer was made by titrating 50 cc. of a 0.20 M KH_2PO_4 solution to pH 7.0 with 0.2 M NaOH and making up to 200 cc. All solutions were made up with water redistilled in an all-pyrex still.

In all studies reported, unless otherwise stated, at least ten experiments were performed on at least three different days to obtain evidence on a given point. When experiments were negative, usually thirty experiments were performed, and sometimes even more. Measurements of animals reported are averages obtained from twenty-five individuals. For such measurements a camera lucida and a stage micrometer were used. The animals to be measured were quickly injected into Dafano's fixative or methyl green and outlined under camera lucida (without pressure from a cover slip). For permanent mounts the animals were fixed in Schaudinn's fixative and stained with Feulgen's nuclear stain, except those of race 1, which were stained with iron hematoxylin.

EXPERIMENTAL

1. *The races of Paramecium used in the investigation.*—Each of the races selected for this study showed characteristics by which it could be distinguished from the others. Members of race 11 were least vigorous, dividing, on the average, but 1.42 times per day; members of race 8 were somewhat slower than the remainder, with a division rate of 2.31 per day; members of races 2, 7, and 9 showed a division rate of about 2.5 per day; and representatives of race 6, 2.71 per day. The data, each from twenty counts and averages for 3-day periods (5 days for race 11), of the number per cubic centimeter in mass cultures are given in Table 1.

All races save 1 and 11 were able to thrive on a suspension of *P. ovalis* in inorganic salt solution as well as in nutrient solution, ten successive transfers being tried in each case. Of the races tested (1, 2, 6, and 11), only members of race 2 were able to thrive on either

TABLE 1

COMPARISON OF THE RACES OF *Paramecium* STUDIED

Race No.	Length in μ	Width in μ	Division Rate per 24 Hours	Percentage Showing Conjugants in Consecutive Trials*	Percentage of Conjugants Viable†
1	183.3±24.2	56.5±8.8	2.47	97.0 (66)	Low
2	187±20.2	59.3±2.3	2.50	88.6 (26)	50 (20)
6	189±15.3	69±7.2	2.71	85.2 (27)	10 (40) 68 (60)
7	199±21.8	77±4.8	2.42	96.4 (28)	25 (40)
8	203±14.6	66±8.2	2.31	84.2 (19)	65 (20)
9	162±26.1	64±4.7	2.54	100.0 (27)	48 (35)
11	171±20.0	49.4±6.0	1.42	100.0 (25)	5.6 (67)

* The numbers in parentheses indicate the number of consecutive trials made.
† The numbers in parentheses are the total number of trials made.

Bacillus subtilis or *Pseudomonas fluorescens* in lettuce; members of race 6 grew luxuriantly on *P. fluorescens* for a time, then died out; members of race 11 grew for a period of three transfers on each of the foregoing bacteria.

Individuals of race 9 were shortest of all, being, on the average, 162 × 64 μ; those of race 11 were not much larger, being 171 × 49 μ; those of race 8 were the longest, being 203 × 66 μ; all of the others were of more or less the same size, individuals of race 7 being a bit more rotund than the others. Members of race 2 were generally filled with many more excretion crystals than any of the others and were, in general, more active even during conjugation.

Paramecia of all the races save 11 show micronuclei clearly; but the number is variable, a finding established as the general rule for this species of *Paramecium* by the careful work of Stranghöner (1932). Generally four or fewer micronuclei were seen; in many individuals only one could be seen; in some individuals of race 6 no micronuclei could be observed, although others from the same culture showed them clearly. Such apparently amicronucleate individuals were also observed by Powers and Mitchell in their stocks (1910). In hundreds of specimens of race 11 examined in all stages—dividing, conjugating, in the normal vegetative stages, as well as in exconjugants—no micro-

nuclei could be observed following the Feulgen technique originally used. But when the time in acid was increased to about 20 minutes and the time in fuchsin sulphurous solution to 24 hours, the micronuclei were plainly visible in all the specimens observed. The macronuclei of some members of race 11 showed fragmentation, a phenomenon occasionally observed in specimens of race 6.

Exconjugants of the different races exhibited variable viability. Races 1, 7, and 11 showed rather low viability, only 25 per cent of the exconjugants of race 7 giving rise to good cultures, and only 5.6 per cent of the exconjugants of race 11. Exconjugants of race 6 showed variable viability. In one series of tests only 10 per cent survived; in another, 68 per cent. Exconjugants of races 8 and 9 showed high viability, 65 and 48 per cent, respectively, in the tests made.

2. *Conjugation tests.*—After subculturing had been carried on for several months, conjugation tests were made. For all of the races except race 2, conditions similar to those already described for race 1 were found to be correlated with conjugation. Twenty to thirty tests were made on consecutive days, and other tests were made later at various times. All cases except in trials on races 9 and 11 gave a few negative results. Thus, in 3 per cent of the trials with race 1, in 14.8 per cent with race 6, in 3.6 per cent with race 7, and in 15.8 per cent with race 8 no conjugants were obtained. However, in some cases the conditions were unfavorable for unknown reasons, and death of a few or of many animals occurred; in other cases the cultures were apparently entirely normal, yet no conjugation occurred. Also, on a variable percentage of days the epidemics in cultures of some races were very weak, only 5–10 per cent being in conjugation; for race 8 in 36.8 per cent of the trials such small epidemics were obtained, for other races the percentage was much lower. Sonneborn (1936) has found a correlation between time since previous nuclear reorganization and conjugation. No evidence indicating a rhythm was here obtained, but the study did not extend over a sufficient period of time. A further study is being made. However, when normal epidemics occurred, they were closely correlated with the depletion of food. At least ten trials in which the animals were washed free of nutrient medium with buffered balanced inorganic salt solution were also made with each clone and the epidemics of conjugation were similar to those in the unwashed controls.

If the paramecia are fed a suspension of *P. ovalis* at the time they are concentrated, the epidemic of conjugation is effectively delayed, the delay depending on the amount of food given. A 1-mm. loopful of a 24-hour growth of *P. ovalis* grown on 2 per cent yeast extract in 2 per cent agar (26° C.), scraped even with the edges, was suspended in 1 cc. of buffered balanced salt solution to make a "standard" suspension. Two drops of such a suspension for the animals from $7\frac{1}{2}$ cc. of a standard culture (third day) did not delay conjugation for more than a few hours; 5 drops, however, produced an easily measurable delay of from 12 to 24 hours, depending upon the race and culture. Ten trials were made with race 6, three for each of the others. Therefore, as in the experiments on race 1 previously reported, here, too, feeding delays the onset of the epidemic of conjugation.

Race 2, tested in the same manner, was the single exception. This race was grown in the standard manner for over 5 months before tests were begun. No conjugants were observed over this entire period. Sixty tests were then made in the standard manner over a period of about a month; all were negative. Examinations at 12-hour intervals were made for 3 days on most cases, and in some cases for a week, to make certain that the epidemic of conjugation was not missed. A sticky state appeared at the time conjugation would have occurred in a conjugating race, but not a single union was observed.

Therefore, experiments were now performed in which each of a number of other factors were varied concomitantly with the change in food supply. Thirty trials were made in each case. Changes in (1) substrate from lettuce (*a*) to 0.1 per cent oat-hay mixture and (*b*) to 0.05 per cent *Agaricus;* (2) pH from 7.0 (*a*) to 6.0 and (*b*) to 8.0, using comparable buffered inorganic salt solutions; (3) salt medium (*a*) to M/60,000 $FeCl_3$ and (*b*) to M/48,000 $AlCl_3$, both of which were found effective incitants by Zweibaum (1912); (4) bacteria used as food from *P. ovalis* (*a*) to *P. fluorescens* and (*b*) to *B. subtilis*, food bacteria being considered important by Chatton and Chatton (1931), were all without effect, for no conjugants appeared. Since observation lasted over 3 days after setting out a given test, it is unlikely that even chance conjugants would have been overlooked.

Jennings (1910) reports successive starvation as effective in *Paramecium aurelia* for induction of conjugation. A series of two and three successive starvations was tried without success. Starved animals kept at 26° C. were also observed, but no conjugation occurred. Trials were also made in which animals were taken from cultures before the maximal population had been reached and 12, 24, and 36 hours after the maximal numbers were reached in standard cultures. All proved negative.

Just at the conclusion of the foregoing series of 320 negative experiments, and when the race had been in subculture for over 7 months, conjugants appeared, at first in small numbers and then in large numbers, both in cultures in lettuce-extract fed *P. ovalis* and in cultures fed *B. subtilis*. They appeared at the time the bacterial food was depleted (when the opacity due to suspended bacteria had disappeared). When such cultures were concentrated twenty fold and set out at 20° C., excellent epidemics in which 60–80 per cent were in conjugation appeared. In a series of twenty-six daily consecutive trials, 11.4 per cent showed no conjugation, 34.6 per cent showed rather poor epidemics, and all the others showed excellent epidemics. The reason for failure of a certain percentage to conjugate is not clear; in only a few cases was injury apparent. The interesting point, however, is that conjugation should suddenly appear and occur under exactly the same conditions found to hold for the other races and which previously had failed utterly to evoke even a single conjugant. It seems most likely that some profound change had taken place in the organism which now enabled it to respond to the change in the environmental conditions. The epidemics were observed to occur for the following 7 months, during which the culture was under observation. When the animals were fed a suspension of bacteria, the epidemic was similarly delayed, as in the experiments on the other races already described.

The experiments on race 2 indicate that (1) the proper internal conditions are necessary to secure conjugation; (2) if these are favorable, conjugation will occur following a sudden depletion of the food supply in race 2, just as in the other races tested; and (3) variations in the environmental conditions other than food will not induce conjugation in this race when the internal conditions are such as to be unaffected by the sudden decrease of the food supply.

In the course of observations of epidemics of conjugation in various races over the last 5 years, in only a few instances were almost all the paramecia in conjugation (usually about 60 per cent were united at a given time); at other times a much smaller number or a much larger number were united at the peak of an epidemic. It seemed possible that some internal change had taken place in members of a given clone such that in a given population some of the subraces were "nonconjugating races," others "conjugating

races." To test this supposition, thirty individuals of race 6 were isolated and cultures were grown of each. When these cultures were tested for conjugation in the usual way, the individuals in all save one (in which abnormal development had occurred) conjugated, and the epidemics were comparable but not better than those usually found in the parent 6 stock. There therefore seemed to be no separation of nonconjugating races, and the failure of some individuals to conjugate must be explained in some other way.

To determine whether individuals not united at the peak of an epidemic would conjugate later, such individuals were picked out and observed for a period of time. Only occasional unions occurred, and observations of stained specimens obtained 12, 24, 48, and 72 hours after isolation from the culture in which conjugation was occurring indicated that no nuclear changes were occurring in the majority of the individuals. A very few showed macronuclear breakdown; these might have been exconjugants.

The nuclear behavior indicates that in all races we are dealing with true conjugation. Conjugants show the characteristic ribbon formation during macronuclear breakdown. Exconjugants fixed and stained 12–24 hours following isolation of conjugants almost invariably show macronuclear breakdown. Only 2 exconjugants out of 100 in race 6 had their macronuclei intact; but in race 9, 20 out of 88 examined had their macronuclei intact. Exconjugants of all other races examined gave the characteristic macronuclear breakdown.

In conclusion it appears that both internal and external environmental factors influence the induction of conjugation. When the internal state was "unfavorable," conjugation was not induced by variation of a number of environmental factors tried. Sonneborn (1937), in his exceedingly interesting analysis of conjugation of members of certain races of *P. aurelia*, has shown that presence of two sexes in a culture is a necessary condition for conjugation. Preliminary trials with the races used here indicate that, with the possible exception of race 2, there is no clear indication of sexual differentiation. These studies will be reported in full at a later date. However, when conjugation occurred, it was found to be correlated with comparable environmental conditions for each of the seven races investigated.

SUMMARY

1. Seven races of *P. multimicronucleatum* were found to conjugate under similar environmental conditions.

2. The main change in the environment correlated with conjugation was the depletion of food after a period of plenty.

3. Feeding suspensions of bacteria to paramecia effectively delays the epidemics of conjugation, the delay depending upon the supply of food given.

4. One of the races which in the beginning did not conjugate under the foregoing conditions was unresponsive to changes in temperature, substrate, pH, $FeCl_3$, or $AlCl_3$ solutions, and bacterial food upon which grown, when each of these was varied concomitantly with decrease in food following successive starvations.

5. This race suddenly began to conjugate, and the epidemics induced occurred under conditions similar to those obtaining in the other races.

LITERATURE CITED

AUSTIN, M. L. 1927. Studies on *Uroleptus mobilis*. I. An attempt to prolong the life cycle. II. The conditions necessary for conjugation. Jour. Exper. Zoöl., **49**:149–216.

BAITSELL, G. A. 1912. Experiments on the reproduction of the hypotrichous Infusoria. I. Conjugation between closely related individuals of *Stylonychia pustulata*. Jour. Exper. Zoöl., **13**:47–78.

BARKER, H. A., and TAYLOR, C. V. 1931. A study of the conditions of encystment of *Colpoda cucullus*. Physiol. Zoöl., **4**:620–34.

CHATTON, M. E., and CHATTON, Mme M. 1931. La Conjugaison du *Paramecium caudatum* determinée expérimentalement par modification de la flore bactérienne associée. Race dites conjugantes et non-conjugantes. Compt. rend. Acad. sci., **193**:206–8.

ENRIQUES, P. 1909. La conjugazione e il differenziamento sessuale negli Infusori. III. Azione dei sali sulle epidemie di conjugazione nel *Cryptochilum nigricans*. Mem. Roy. acad. sci. Inst. Bologna, Ser. VI, **6**:463–500.

GIESE, A. C. 1935. The role of starvation in conjugation of *Paramecium*. Physiol. Zoöl., **8**:116–25.

JENNINGS, H. S. 1910. What conditions induce conjugation in *Paramecium?* Jour. Exper. Zoöl., **9**:279–98.

MAST, S. O. 1917. Conjugation and encystment in *Didinium nasutum* with especial reference to their significance. Jour. Exper. Zoöl., **23**:335–59.

MAUPAS, E. 1889. La Rajeünissement karyogamique chez les ciliés. Arch. zoöl. expér. et generale, **7**:149–517.

POWERS, J. H., and MITCHELL, C. 1910. A new species of *Paramecium* (*Paramecium multimicronucleata*) experimentally determined. Biol. Bull., **19**:324–31.

PRANDTL, H. 1906. Die Konjugation von *Didinium nasutum* F. M. Archiv f. Prot., **7**:229–58.

SONNEBORN, T. M. 1936. Factors determining conjugation in *Paramecium aurelia*. I. The cyclical factor: the recency of nuclear reorganization. Genetics, **21**:503–14.

———. 1937. Sex, sex inheritance and sex determination in *Paramecium aurelia*. Proc. Natl. Acad. Sci., **23**:378–85.

SONNEBORN, T. M., and COHEN, B. M. 1936. Factors determining conjugation in *Paramecium aurelia*. II. Genetic differences between stocks or races. Genetics, **21**:515–18.

STRANGHÖNER, E. 1932. Teilungsrate und Kernreorganizationsprozess bei *Paramecium multimicronucleatum* Powers and Mitchell. Arch. f. Prot., **78**:302–60.

ZWEIBAUM, J. 1912. Les Conditions necessaires et suffisantes pour la conjugaison du *Paramecium caudatum*. Arch. f. Prot., **26**:275–393.

REPRODUCTION OF PARAMECIUM IN OLD CULTURE MEDIUM

(Three figures)

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ALLEE (1931) states that "there is a general ecological assumption that the accumulation of the waste products of a given species in their habitat tends, with most animals, to limit the time of their occupancy, at the same time preparing the way for another species to come in." In his discussion of bacteria Henrici (1928) says: "The idea that growth is limited by the accumulation of some toxic substance is the one that seems to be most generally accepted, though the evidence for it is far from being convincing." The idea contained in these two statements relative to the role of waste products has been restated or assumed in most protozoan studies of the last forty years.

In the first careful work on the subject Woodruff (1911) found an effect of waste products when from 1 to 4 animals had lived in about 0.5 cc. of medium for 24 hours. Using volumes of media, periods of time, and numbers of animals comparable to those employed by Woodruff, Myers (1927), Calkins (1926), Greenleaf (1926), Peterson (1929), Di Tomo (1932), and Beers (1933) obtained depressing effects of excretory products on protozoan division-rates. With reference to bacteria-free cultures of *Glaucoma*, Jahn (1934) says: "The fact that the supply of *N* compounds is not greatly decreased before growth stops indicates that cessation of growth is caused by the accumulation of waste products." Gause (1934) in his extensive experiments with protozoan populations accepts the conclusions of Woodruff concerning the effects of waste products on division-rate and states that he has eliminated the complicating influence of these substances by changing the medium in his cultures every 24 hours.

A few other investigators have reported results which differ considerably from those referred to above. Dimitrowa (1932) found that a very small amount of old culture medium added to fresh medium caused *Paramecium* to divide faster than they did in nonconditioned medium. Phelps (1936) obtained populations of *Glaucoma* grown in pure culture on yeast extract as dense as 500,000 per cubic centimeter. He states that concentrations of animals more than one thousand times greater than those observed in other investigations were obtained before the effects of excretory products on division-rate were noticed. In considering these differences, Phelps made two suggestions. In some cases he considered that the difference might be due to the fact that, as a population of mixed bacteria were growing along with the protozoa, the waste products of these bacteria might have had a depressing effect on the protozoan division-rate. He also suggested that different kinds of food might cause the same protozoan to produce excretory products quite different in nature and effects.

Recently, Taylor and Strickland (1938) have been able to grow *Colpoda duodenaria* in 500-cc. cultures of balanced salt solution with a single kind of bacterium as the source of food, with uniform growth-rates. They have found that excretion products, accumulating in the medium for 4 months, do not affect the growth curves.

In previous studies (Johnson, 1933 and 1937) it was pointed out that experiments were needed to test the relative roles of food and waste products on division-rate in protozoan populations feeding on bacteria. To do this has been the aim of these studies.

MATERIALS AND METHODS

The experimental animals were taken from a clone of *Paramecium multimicronucleata* which has been maintained in this laboratory for some time. The paramecia were grown in a nonnutritive balanced salt solution on suspensions of a strain of the bacterium *Pseudomonas fluorescens* obtained from the Stanford University bacteriology department. The bacteria were grown on agar plates. The method of making the salt solution has been described elsewhere (Johnson, 1933). A platinum loop with an inside diameter of 1.5 mm. was used to measure the suspensions. The paramecia were cultured in 5 and 10 cc. of the liquid with 1 loopful of bacteria for each 5 cc. of liquid as the standard suspension (*X* concentration). Preliminary experiments using 1 *Paramecium* in 2 drops of different bacterial densities gave results which indicated that the density of bacteria used in the standard suspensions was about the optimum for this ciliate at 20° C. In the 5-cc. cultures, 80 paramecia were introduced at the beginning of the experiment. In the 10-cc. cultures, 160 animals were used.

The culture dishes were kept in moist chambers at constant temperatures. In some experiments the temperature was 20° C.; in others, 26° C.

The cultures were centrifuged for counting. A glass rod with a ball on one end was used as a plunger to trap the paramecia in the bottom of the centrifuge tube. In this manner it was possible to trap all of the paramecia in approximately ½ cc. of the culture fluid. The remainder of the liquid was poured from the tube, and in the case of the experimental cultures was used with ½ cc. of fresh medium for the next day's culture fluid. In every case the liquid poured from the centrifuge tube was examined for paramecia. The control cultures were made up each day from fresh medium. By this method the experimental cultures consisted of 4½ cc. of old medium plus ½ cc. of fresh medium. It was assumed in the beginning that the experimental culture medium would accumulate more and more waste material as it was used over and over again. To take account of any possible effect of the ½-cc. dilution each day, other experiments were devised which will be described later.

For the actual counts the paramecia trapped in the centrifuge tube were transferred to a large depression slide, and the counts were made with a micropipette. The animals for each experiment were taken from the cultures of the preceding day.

To insure the same amount of food in both control and experimental cultures, where ½ cc. of liquid was lost from centrifuging, a dense suspension of bacteria was made by adding 5 loopfuls of bacteria to 2½ cc. of salt solution; and then ½ cc. of this suspension was added to 4½ cc. of culture fluid in each case. In those experiments where no liquid was added to the old culture medium, attempts were made to fill the loop with the same amount of bacteria each time.

Unless stated otherwise, the culture fluid was filtered through a Chamberland-Pasteur porcelain filter. The salt solution was buffered (as previously described) to a pH of 6.9–7.0, and the pH of the culture fluid was tested each time before the fluid was discarded.

Any deviations from the above-described methods will be stated in the discussion of the experiments.

EXPERIMENTAL

At the outset the best approach to the problem seemed to be in the use of the same medium over and over, filtered each day to remove all the bacteria. In this way the control series and the experimental series with the old medium could be kept the same with respect to the amount of food.

TABLE 1

USE OF FILTERED OLD MEDIUM

Days	Division-Rates		Days	Division-Rates	
	Control	Experimental		Control	Experimental
1	2.04	.....	11	1.63	1.29
2	1.96	1.85	12	1.10	1.20
3	2.03	2.17	13	1.25	1.16
4	2.46	2.11	14	2.25	2.15
5	1.91	1.28	15	2.11	1.93
6	1.87	1.34	16	1.76	1.09
7	2.30	2.06	17	2.21	2.29
8	1.28	1.03	18	1.04	1.62
9	1.40	1.83	19	2.07	2.00
10	2.03	1.60			
			Mean	1.81	1.66

Probability*: Control versus experimental, $P = .0746$

* All calculations by the method of "Student" (1925).

Table 1 shows the results of such an experiment over a 19-day period. The fission-rates given here are the averages of two controls and two experimentals each day. As approximately 1 cc. of fluid was lost in the candle, the media from the two 5-cc. experimental cultures were mixed and filtered together. Each culture lost ½ cc. of medium in centrifuging. Thus, each culture lost altogether 1 cc. of medium. This loss was made up with fresh medium. The standard suspension of bacteria was used, and the cultures were kept at 20° C. New cultures were seeded with animals from the same type culture of the preceding day.

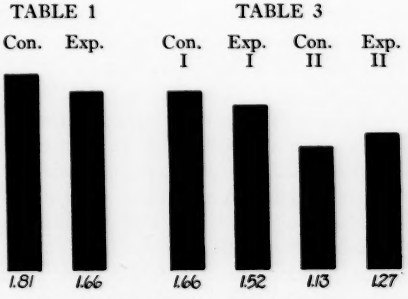

FIG. 1.—Showing the mean fission-rates from Tables 1 and 3.

The mean fission-rate of the controls is slightly higher than the experimentals, but the difference is not statistically significant. On the basis of many of the results cited above, it was anticipated that there would be a gradual decrease in the fission-rate of the experimental series. This, however, was not the case. It seemed that either there was no pronounced effect of waste products for this period of time or the filter removed such substances and prevented their effects. Eijkman (1907) and Curran (1925) found

that growth-inhibiting substances from bacterial cultures would not pass through porcelain filters, at least not in the early stages of the filtering process. So it seemed that this sort of thing might have happened here.

In the preliminary experiments using small volumes of the standard suspension (in the ratio of one organism for each drop of medium) the medium appeared absolutely clear after 24 hours. Apparently, the paramecia had eaten most of the bacteria. Because of this it seemed that it might be possible to use the same medium over and over, unfiltered, without producing bacterial crowding of the protozoans.

Before describing such experiments it seems advisable to refer to a difficulty encountered in the use of unfiltered salt solution. Both with control and with experimental cultures it was possible to obtain consistent results for several days, and then the fission-rate would drop and in some cases the cultures would have fewer organisms than the original seeding at the end of 24 hours.

TABLE 2

COMPARING FILTERED AND UNFILTERED MEDIUM AT 26° C.

Days	Filtered	Unfiltered	Days	Filtered	Unfiltered
1	2.02	0.72	15	2.55	2.30
2	1.99	1.60	16	2.22	(62)*
3	2.05	0.95	17	2.16	0.86
4	2.18	1.87	18	1.77	1.09
5	2.34	2.22	19	2.25	2.20
6	2.02	1.82	20	2.24	2.02
7	2.49	2.23	21	2.18	1.50
8	2.16	1.92	22	2.21	(17)*
9	2.07	2.07	23	2.48	1.68
10	1.66	1.10	24	2.87	0.03
11	1.87	(73)*	25	2.70	2.47
12	2.18	2.01	26	2.64	2.49
13	2.29	1.75	27	2.12	1.83
14	2.41	2.17	28	2.25	0.41

* The actual number of organisms is given in parenthesis. In cases where the division-rate fell below 0.00, the deficiency in animals was made up with paramecia taken from the filtered cultures, so that the seeding for the following day was again 80 animals.

Table 2 gives results with two control series, one using new filtered salt solution, the other unfiltered salt solution. Increase in the filtered medium was quite regular, but it was very inconsistent in the unfiltered medium. In the experiments cited in Table 1 the control medium was filtered each day simply as a control procedure. On the basis of results given in Table 2 and of other results, it was decided to filter all the salt medium used in subsequent experiments.

In previous work (cf. Johnson, 1933 and 1936) this difficulty with the unfiltered balanced salt solution did not exist. In this work Merck's C.P. chemicals were used in preparing the salt solution. It seems probable that there were traces of heavy metals or other substances in the salt solution, as prepared, which were adsorbed by the filter. When filtered, the salt solution was quite satisfactory for culturing *Paramecium*.

In Table 3 are given the results of two series of experiments conducted at different times under the same conditions. In Series I the controls have a slightly higher average fission-rate; in Series II the experimentals have a slightly higher average fission-rate.

Taken together, these two series of experiments do not indicate an important cumulative effect of waste products in the old medium. When double the standard suspension was used in these experiments, the results did not indicate a very marked crowding effect of

TABLE 3

USE OF UNFILTERED OLD MEDIUM AND 2X CONCENTRATION OF BACTERIA AT 20° C.

Days	Series I		Series II	
	Control	Experimental	Control	Experimental
1	1.62	.....	1.32	.....
2	1.12	1.00	1.71	1.06
3	0.95	1.57	0.97	1.70
4	0.88	1.08	1.87	1.74
5	1.27	1.36	1.71	1.03
6	1.53	1.66	0.61	1.39
7	2.05	1.70	0.50	1.42
8	1.91	1.66	0.23	0.29
9	0.82	1.23	1.62	2.32
10	1.59	1.68	1.09	1.14
11	2.16	1.72	1.60	1.21
12	1.88	1.72	1.18	1.13
13	1.97	1.71	0.97	0.84
14	1.98	1.77	0.92	0.99
15	2.03	1.81	1.47	0.91
16	1.45	1.11	1.09	0.84
17	1.50	1.30	0.94	0.39
18	1.50	0.35	1.56	0.87
19	1.31	1.31	0.83	0.96
20	1.51	1.28	1.27	1.22
21	1.88	1.62	1.48	1.48
22	1.77	1.55	1.00	1.86
23	1.75	1.78	0.75	1.61
24	1.89	1.81	0.76	1.33
25	1.97	1.67	1.06	1.56
26	1.94	1.72	1.36	1.43
27	1.97	1.74	1.01	1.52
28	1.80	1.92	0.89	1.45
29	1.88	1.73	0.43	1.73
30	2.12	1.87	0.64	1.72
31	1.93	1.60	1.12	1.32
32	1.88	1.37	1.68	1.69
33	1.91	1.65	1.94	1.71
34	0.81	1.35	.....	.....
Mean	1.66	1.52	1.13	1.27
Probability	Control versus experimental, Series I, $P = .0163$			
	Control versus experimental, Series II, $P = .1615$			

bacteria, if any. Here, as before, the new cultures were seeded from the same type culture of the day before.

Similar results were obtained in a series of cultures using 10 cc. of the standard suspension with initial seedings of 160 paramecia each day. In this case, also, the new

cultures were seeded from the same type culture of the preceding day. By using this larger volume of medium, it was possible to reduce the dilution of the old medium each day. Here the amount of old medium saved each day was 9½ cc., while in 5-cc. cultures 4½ cc. were saved. This difference did not affect the nature of the results which are given as Series I in Table 4.

TABLE 4

RESULTS WITH 5- AND 10-CC. CULTURES AT 20° AND 26° C.

Days	Series I (10 Cc. at 26° C.)		Series II (5 Cc. at 20° C.)	
	Control	Experimental	Control	Experimental
1	1.57	.....	1.10	.....
2	1.38	1.44	1.07	0.93
3	1.96	1.75	1.23	1.84
4	2.15	2.62	1.07	1.45
5	2.35	2.72	1.03	2.34
6	2.29	2.31	1.35	0.56
7	2.30	2.49	1.75	1.61
8	2.58	2.56	2.01	2.07
9	2.51	2.63	1.95	2.19
10	1.92	2.46	2.29	2.22
11	2.42	2.47	2.21	1.84
12	2.24	2.73	2.06	1.73
13	2.33	2.56	1.86	1.56
14	3.03	2.94	1.95	1.81
15	2.67	2.60	1.99	1.88
16	2.08	2.64	1.99	1.67
17	2.59	2.61	1.26	1.07
18	2.99	2.40	1.12	0.79
19	2.77	2.92	1.60	1.42
20	2.61	2.67	1.79	1.78
21	2.80	2.93	1.76	1.73
22	2.29	2.24	1.80	1.49
23	2.70	2.84	2.39	1.71
24	2.62	2.51	1.85	1.83
25	2.48	1.75	1.77	1.22
26	2.04	2.51	1.38	1.41
27	2.14	2.12	1.35	1.19
28	2.59	2.89	1.75	1.76
29	2.80	2.51	1.83	1.43
30	2.89	2.56	1.71	1.30
31	2.27	1.34	.....	.....
Mean	2.42	2.46	1.69	1.53

Probability: Control versus experimental, Series I, $P = .1166$; Control versus experimental, Series II, $P = .1095$

In all the experiments described so far, the new cultures were seeded each day with animals from the same type of culture of the preceding day. In the experiment reported as Series II in Table 4, both experimental and control dishes each day were seeded with animals from the control dish of the day before. The failure to obtain a significant difference between the experimentals and controls in this case seems to indicate that the

absence of such a difference in the preceding experiments was not due to some acclimating process in the organisms growing in the old medium.

Results comparing division-rates of cultures in X and $2X$ concentrations of bacteria at 26° C. and with all the cultures seeded from the controls of the preceding day are given in Table 5. The difference between the experimentals and controls in the $2X$ concentrations is not significant. In the case of the X concentrations, the higher fission-rate in the controls would ordinarily be taken as significant. A comparison of the two control series shows a significantly higher rate in the $2X$ concentrations. When the two experimental series are compared, a higher mean fission-rate in the $2X$ group is found, but the difference is not significant. During two of the last three days of the experiment the division-rates in the $2X$ group of experimental dishes were very low. The reason for this is not known. However, when the 2 experimentals are compared for the first 27

TABLE 4

TABLE 5

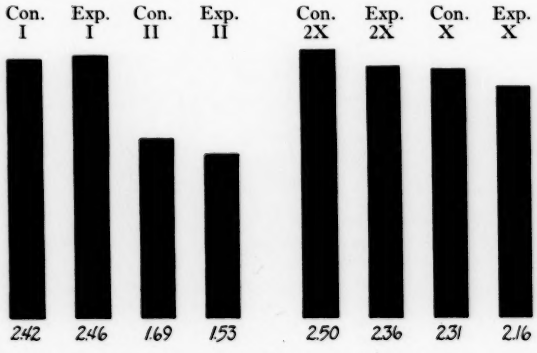

FIG. 2.—Showing the mean fission-rates given in Tables 4 and 5

days, the $2X$ group has a higher rate, with a probability of .0013. Apparently, at 26° C. the $2X$ concentrations of bacteria are nearer the optimum than are the X concentrations.

Most of the earlier work, referred to above, in which the effects of waste products on reproduction were described, was carried out using hay tea or some other nutrient fluid as the culture medium. It seemed desirable to have a series comparing results obtained from hay cultures and the balanced salt cultures.

The hay medium used in these experiments was made by boiling for 3 or 4 minutes 2½ gm. of Timothy hay in a liter of buffered balanced salt solution. After cooling, the medium was passed through a porcelain filter. It was then measured out into test tubes and autoclaved. Thus, the hay medium for the whole series was uniform. In other respects the procedure was unchanged. The hay cultures, both fresh and old, were bacterized with 1 loopful of bacteria each day, just as the control cultures were.

There is no significant difference between the fission-rates of these three series. In general it can be stated that the results in the salt control series varied less than those in the two hay series.

With no definite inhibiting effect of accumulated waste products apparent in the work up to this point, there still seemed to be a possibility that the small dilution of the old

medium each day, owing to the nature of the technique, might waive such effects. Two procedures to check this possibility were developed. One was to use the same medium over and over in decreasing volumes each day and thus avoid any dilution. The other was to use liquid in which large numbers of paramecia had been growing for a long time.

TABLE 5

RESULTS WITH X AND $2X$ CONCENTRATIONS OF BACTERIA

Days	$2X$ Concentration		X Concentration	
	Control	Experimental	Control	Experimental
1	2.62	.....	2.66	.....
2	2.39	2.35	2.26	2.22
3	2.71	2.75	2.09	1.43
4	1.68	1.87	1.74	1.63
5	2.14	2.06	2.24	2.04
6	2.33	1.44	2.56	1.99
7	2.88	3.48	2.91	1.91
8	3.00	2.58	2.19	1.57
9	2.55	2.56	2.25	1.15
10	2.74	2.75	2.65	2.55
11	3.24	3.29	3.41	2.67
12	3.01	3.27	2.72	2.69
13	2.38	2.46	1.93	1.56
14	2.47	2.32	2.21	2.40
15	3.02	2.87	2.75	2.51
16	2.08	2.04	2.03	1.95
17	2.85	2.85	2.52	2.53
18	1.96	2.44	1.95	2.16
19	2.02	1.90	2.25	2.03
20	2.74	2.72	2.39	2.41
21	2.70	2.88	2.99	2.89
22	2.27	2.33	2.39	2.41
23	2.54	2.83	1.76	2.15
24	2.36	2.40	1.49	1.45
25	2.38	2.16	1.97	2.13
26	2.89	2.74	2.85	2.67
27	2.32	1.52	1.73	2.38
28	2.44	2.48	2.62	2.40
29	2.86	1.10	2.49	2.69
30	1.71	0.15	1.89	2.02
31	2.43	2.24	2.24	2.10
Mean	2.50	2.36	2.31	2.16

Probability:
- $2X$ control versus $2X$ experimental, $P = .1095$
- X control versus X experimental, $P = .0278$
- $2X$ control versus X control, $P = .0026$
- $2X$ experimental versus X experimental, $P = .1615$

The control culture reported in Table 7 was started with 10 cc. of the standard suspension and seeded with 160 paramecia. The liquid ($9\frac{1}{2}$ cc.) from this culture was used as the experimental medium on the second day and was seeded with 152 paramecia. The control culture of the second day had the same amount of fluid and the same number of

organisms. On each succeeding day the volume of the cultures was reduced ½ cc., and the seeding of organisms was 8 less than the preceding day. The volume of the cultures on the last day was 2 cc. With the volumes of the cultures and the seedings of animals

TABLE 6

COMPARING BACTERIZED HAY MEDIUM WITH SALT CONTROL AT 26° C.

Days	Salt Control	Fresh Hay	Old Hay	Days	Salt Control	Fresh Hay	Old Hay
1	2.19	2.74	.....	17	1.84	1.42	1.97
2	2.24	2.29	1.46	18	1.91	1.14	2.05
3	2.13	2.93	0.66	19	2.90	1.03	2.54
4	1.97	2.49	1.28	20	2.77	1.95	2.18
5	1.79	2.87	1.72	21	2.15	2.39	1.73
6	2.16	2.35	2.21	22	2.17	3.09	2.66
7	2.76	3.02	2.85	23	1.88	2.09	2.53
8	1.99	2.82	2.56	24	2.51	0.88	3.13
9	1.81	1.56	2.38	25	2.67	3.00	2.21
10	2.51	3.04	2.66	26	2.38	3.23	2.41
11	2.34	2.88	2.46	27	2.65	3.22	3.01
12	1.98	2.41	2.03	28	2.38	1.44	2.57
13	2.33	2.62	1.86	29	2.74	2.03	3.46
14	1.53	2.77	3.34	30	2.32	2.71	2.57
15	1.84	1.49	2.20				
16	1.62	2.25	2.18	Mean	2.21	2.32	2.30

Probability: Fresh hay versus old hay, $P = .5203$; Old hay versus salt control, $P = .4237$; Fresh hay versus salt control, $P = .2301$

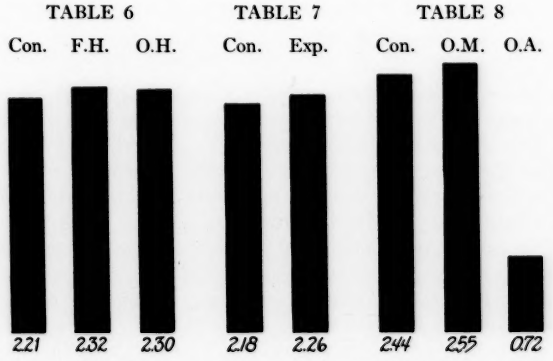

FIG. 3.—Showing the mean fission-rates given in Tables 6, 7, and 8

proportional throughout, the fission-rates were quite constant. There was no significant difference between the experimentals and the controls for the experiment as a whole.

In order to have an old medium in which paramecia had been growing and living in large numbers for a long time for use in these experiments, two 1-liter flasks of buffered

salt solution were seeded, each with a single *Paramecium*. One of these liter cultures has been inoculated with 10 heaping loopfuls of *P. fluorescens* every day since its beginning. The other one has been inoculated with the same amount of bacteria every other day. In each case the population gradually increased until a stationary level in numbers was reached. In the first case a level of approximately 225 organisms per cubic centimeter was reached after 20 days, and has been maintained for over 4 months. In the second case a level of approximotely 175 was reached after 29 days, and here also the numbers have remained almost constant. A detailed study of these populations will be the subject of another paper.

Five cubic centimeters of medium from one of the liter cultures which had been freed of all paramecia were used as the old medium in each of the four series given in the upper section of Table 8. A standard loopful of bacteria was used in each culture. The seedings of both the control cultures and the old-medium cultures were made from the control

TABLE 7

USE OF UNDILUTED OLD MEDIUM AT 26° C.

Days	Control	Experimental	Days	Control	Experimental
1.....	2.14	.....	10.....	2.37	2.58
2.....	1.73	1.92	11.....	2.34	2.11
3.....	2.27	2.57	12.....	1.89	1.15
4.....	2.21	2.26	13.....	2.12	2.13
5.....	2.31	2.42	14.....	1.93	2.44
6.....	2.25	2.49	15.....	2.49	2.35
7.....	2.29	2.82	16.....	2.18	2.06
8.....	2.13	2.32	17.....	2.25	2.22
9.....	2.19	2.32			
			Mean....	2.18	2.26

Probability: Experimental versus control, $P = .2886$

culture of the preceding day. The old medium varied in age from 29 to 108 days; yet there was no significant difference between the controls and the old-medium cultures. Quite a different situation existed in the fresh salt-solution cultures seeded with paramecia taken from the liter flasks. In all four cases the fission rates were less than 1.00. The results obtained using control animals in the old medium indicated that there was nothing in the old fluid which inhibited growth and division. The difference here seems to be due to a difference in physiological state between the control animals and those in the liter cultures. Actually, the paramecia in the liter cultures were in a state of semistarvation. By taking a 5-cc. sample from each of the liter flasks after the daily count and feeding, and checking the increase at the end of 18 hours, an indication of the normal rate of increase in the flasks was obtained. In the case of the sample taken from the liter flask which was fed daily, the increase after 18 hours was 0.22. In the other sample from the flask which was fed every other day, the rate of increase after 18 hours was 0.32. The 5-cc. control cultures were fed 1 loopful of bacteria daily. The 1,000-cc. cultures were fed 10 loopfuls of bacteria either daily or every other day. The results given in the lower section of Table 8 show that the division-rates of the paramecia taken from the liter cultures had not been permanently lowered. In one case, Series II, the

cultures were continued for 5 days with the medium changed daily. On both the fourth and fifth days the liter-animal cultures were about equal to the controls. In another case, Series III, after the first day the liter animals were placed in 5 cc. of medium taken from the liter flasks, and this was used over again on the succeeding days. Here, also, the fission rates of the experimentals came up to that of the controls. These results seem to indicate that a period of acclimation is necessary after *Paramecium* has been in a state of semistarvation for a long period of time.

TABLE 8

USE OF OLD MEDIUM FROM 1-LITER CULTURES AT 26° C.

Series	Age in Days	Control	Control Animals in Old Medium	Animals from Old Cultures in Fresh Salt
I	29	2.52	2.52	0.55
II	43	2.54	2.49	0.59
III	49	2.48	2.35	0.89
IV	108	2.24	2.86	0.88
Mean		2.44	2.55	0.72

Probability: Control versus control animals in old medium, $P = .5740$
Control versus animals from old culture, $P = .0000$

Days	From Series II (Medium Changed Daily)		From Series III (Old Medium Used Over)	
	Control	Experimental	Control	Experimental
1	2.54	0.59	2.48	0.89
2	2.64	0.52	2.50	1.12
3	2.05	1.79	2.41	2.10
4	2.90	2.61	2.01	2.16
5	2.58	2.57	2.09	2.13

To obtain bacteria-conditioned medium, a normal loopful of bacteria was added to a dish containing 5 cc. of the salt solution for a number of days. This medium was then used for culturing *Paramecium*. Table 9 shows the results obtained using such conditioned medium of different ages. All the bacteria-conditioned medium used inhibited growth of the paramecia, the older the medium the greater the inhibition. In most of the 30-day cultures some of the paramecia died. Both the filtering through a porcelain filter and the autoclaving of this medium improved it some, but even then it was quite inferior to the normal salt solution. Filtering showed that this depressing effect was not entirely due to bacterial crowding of the paramecia.

Bacterial counts on control cultures at the end of the 24-hour period showed that the original density had been reduced to from 2 to 16 per cent of the original density in different counts made. Thus, most of the bacteria were being used up each day. Under

such conditions, and with some of the remaining bacteria removed in the centrifuging, the old culture medium which was used over and over would not accumulate excess bacteria rapidly. It seems probable, both from the appearances of the cultures and from the amount of bacteria inoculated for the number of organisms, that the old medium used from the liter cultures was relatively free from bacteria at the time it was removed.

TABLE 9

USE OF BACTERIA-CONDITIONED MEDIUM

Length of Conditioning (Days)	Conditioned	Control
15	1.47	2.69
20	0.77	2.32
20	0.14	2.59
30	(17)*	2.47
30	(78)*	2.24
30	(61)*	2.56
30	(64)*	2.01
30 (hay)	0.14	2.32
30 (filtered)	1.21	2.35
30 (autoclaved)	0.99	2.20
30 (1 cc.+4 cc. of salt solution)	1.42	2.09

* The actual numbers of *paramecia* are given in parentheses.

TABLE 10

SUMMARIZING TABLE

Table No.	Mean Division-Rates		Table No.	Mean Division-Rates	
	Control	Experimental		Control	Experimental
I	1.81	1.66	V (2X)	2.50	2.36
III (1)	1.66	1.52	V (X)	2.31	2.16
III (2)	1.13	1.27	VI	2.21	2.30
IV (1)	2.42	2.46	VII	2.18	2.26
IV (2)	1.69	1.53			

Probability: Control versus experimental, $P = .3944$

DISCUSSION

The results described above are not in agreement with the results obtained by most of the earlier workers on protozoan populations concerning the effects of waste products on reproduction. They are, however, essentially like the recent results of Phelps (1936) and of Taylor and Strickland (1938). In only two of the nine paired series reported were the control fission-rates significantly higher than the experimental ones. A comparison of the mean division-rates of the experiments as a whole is given in Table 10. The

P-value given here indicates no significant difference in division-rate between the cultures grown in fresh medium and those grown in old medium for the experiments as a whole.

The investigators who reported an inhibiting effect of waste products on reproduction in from 24 to 48 hours used hay tea or some other nutrient medium with unknown mixed bacteria as the source of food. In these experiments when hay medium was used over and over again for cultures but with a fresh supply of a known kind of bacterium added each day, reproduction comparable to the daily fresh controls was obtained. In view of these findings it seems probable that workers who described effects of waste products in from 24 to 48 hours were dealing with a situation in which the protozoans had utilized most of the bacteria suitable as food for them in that period of time and thus exhibited a declining fission-rate because of the lack of food. The fact that at the end of a 48-hour period numerous bacteria are present in a protozoan culture, when the kinds of bacteria present are unknown, is no proof that those bacteria are suitable as food for the particular protozoan being cultured.

The ideas suggested by Phelps (1936) should not be ignored. It has been shown here that medium conditioned with a single kind of bacteria for a number of days does inhibit reproduction of *Paramecium*. His suggestion, that the inhibiting effects described by several investigators might have been due to the accumulated wastes of the mixed bacteria in the cultures, should be tested with actual experiments. It would appear, from this work, that such effects would not be noticeable in such short periods of time. His second suggestion, that with different sources of food the same protozoan might produce entirely different waste products, also needs testing.

The results of Dimitrowa (1932) might be explained by the hypothesis that she changed the bacterial flora significantly when she added small amounts of old culture liquid to new medium.

The conclusion reached here, that, with the culture conditions used, the accumulated waste products in old culture medium did not significantly lower the reproductive rate of *Paramecium*, does not preclude entirely such effects under other conditions or for criteria other than reproduction-rate. Such questions as "Are the waste products of such cultures, in the main, volatile substances which readily escape from the medium?" "Are these waste substances very unstable materials which are changed by the associated organisms in the culture and thus rendered unharmful?" and "Are the waste products adsorbed by the dead organisms or secretions of the organisms in the culture and thus made ineffective?" are suggested; but they cannot be answered here.

The changes in the H-ion concentration of all of the cultures studied were slight and consistent. The medium tested after 24 hours, old medium tested after 30 days of use, old medium taken from the liter cultures, and 30-day-old bacteria-conditioned medium tested pH 7.2 in all cases.

As was to be expected, cultures grown at 26° C. had a consistently higher fission-rate than those grown at 20° C.

SUMMARY

1. For the conditions described, there was no significant inhibiting effect on the reproductive rates of *P. multimicronucleata* grown in old culture medium with the waste products supposedly accumulating.

2. Similar results were obtained both in the use of bacterized balanced salt medium and in bacterized hay medium.

3. The seeding of the old-medium cultures with organisms from the preceding day's old-medium culture over and over gave results which were very comparable to those obtained in old-medium cultures which were seeded each day from control cultures started the preceding day.

4. Paramecia which have lived in a condition of semistarvation for a period of time and which have been reproducing with a very low division-rate will, when placed in medium containing adequate food, resume a normal rate of division after a period of 2 or 3 days.

5. Medium conditioned with the bacterium *P. fluorescens* for 15–30 days when used for the culture of *Paramecium* definitely inhibits reproduction.

6. Changes in H-ion concentration in all of the cultures were slight and consistent.

7. Higher fission-rates were obtained at 26° C. than at 20° C., and $2X$ concentration of bacteria was nearer the optimum at 26° C. than X concentration.

LITERATURE CITED

ALLEE, W. C. 1931. Animal aggregations. Chicago: University of Chicago Press. Pp. 431.

BEERS, C. D. 1933. The relation of density of population to rate of reproduction in the ciliates *Didinium nasutum* and *Stylonychia pustulata*. Arch. f. Protistenk., **80**:36–64.

CALKINS, G. N. 1926. Biology of the protozoa. Philadelphia: Lea & Febiger. Pp. 625.

CURRAN, H. R. 1925. The growth of bacteria. Thesis, Cornell University.

DIMITROWA, A. 1932. Die fördernde Wirkung der Exkrete von *Paramoecium caudatum* Ehrbg. auf dessen Teilungsgeschwindigkeit. Zoöl. Anz., **100**:127–32.

DI TOMO, M. 1932. Richerche sul comportamento di *Paramecium caudatum* in un dato volume di cultura liquida. Boll. di zool., **3**:137–40.

EIJKMAN, C. 1907. Über die Ursache der Wachstumhemmung in Bacterienkulturen. Deutsche med. Woch., **33**:265–66.

GAUSE, G. F. 1934. The struggle for existence. Baltimore: Williams & Wilkins Co. Pp. 163.

GREENLEAF, W. E. 1926. The influence of volume of culture medium and cell proximity on the rate of reproduction of Infusoria. Jour. Exper. Zoöl., **46**:143–69.

HENRICI, A. T. 1928. Morphologic variation and the rate of growth of bacteria. Springfield, Ill.: C. C. Thomas. Pp. 194.

JAHN, T. L. 1934. Problems of population growth in the protozoa. Collecting Net, **9**:34–41, 47–48.

JOHNSON, W. H. 1933. Effects of population density on the rate of reproduction in *Oxytricha*. Physiol. Zoöl., **6**:22–54.

———. 1936. Studies on the nutrition and reproduction of *Paramecium*. *Ibid.*, **9**:1–14.

———. 1937. Experimental populations of microscopic organisms. Amer. Nat., **71**:5–20.

MYERS, E. C. 1927. Relation of density of population and certain other factors to survival and reproduction in different biotypes of *Paramecium caudatum*. Jour. Exper. Zoöl., **49**:1–43.

PETERSEN, W. 1929. The relation of density of population to rate of reproduction in *Paramecium caudatum*. Physiol. Zoöl., **2**:221–54.

PHELPS, AUSTIN. 1936. Growth of protozoa in pure culture. II. Effect upon the growth curve of different concentrations of nutrient materials. Jour. Exper. Zoöl., **72**:479–96.

"STUDENT," 1925. New tables for testing the significance of observations. Metron, **5**:105–20.

TAYLOR, C. V., and STRICKLAND, A. G. R. 1938. Reactions of *Colpoda duodenaria* to environmental factors. I. Some factors influencing growth and encystment. Archif. f. Protistenk. (in press).

WOODRUFF, L. L. 1911. The effect of excretion products of *Paramoecium* on its rate of reproduction. Jour. Exper. Zoöl., **10**:551–81.

STUDIES ON THE PHYSIOLOGY OF AMOEBA. II. THE ALLELOCATALYTIC EFFECT IN AMOEBA CULTURES FREE OF BACTERIA

(Four figures)

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SINCE the phenomenon of allelocatalysis was first described by Robertson (1921), it has been intensely discussed in the protozoölogical literature; however, no definite conclusion has been reached in this discussion. As the earlier results were dealt with in detail by Allee (1931), Beers (1933), and Johnson (1933), I may confine myself to giving a brief summary, which is necessary for clearing up the present state of the problem. The earlier publications on allelocatalysis may be classed under three headings. The first confirms the phenomenon of allelocatalysis. Besides Robertson's work on *Enchelys farcimen* (1921), *Colpoda* sp. (1924 *a*, *b*), and the long series of his publications dating up to 1927, this group comprises only Yocom's paper on *Oxytricha* (1927) and Petersen's on *Paramaecium caudatum* (1929). Petersen's results deserve our special attention, as she demonstrated the importance of the relation between the volume of the animals and that of the medium. Her results, while negative for cultures in 1 drop of nutrient solution, were positive in media of 20–40 drops.

The second group, including the work with negative result, is far larger. Cutler and Crump (1923) showed that in *Colpidium colpoda* the division rate of cultures with a larger initial number of individuals does not exceed that of cultures with smaller initial numbers. In 1925 (in answer to the objections of Robertson [1924]), they confirmed their result for well-washed infusoria. Greenleaf (1926) investigated *P. aurelia*, *P. caudatum*, *Pleurotricha lanceolatum*, and *Stylonychia pustulata* and arrived at negative conclusions in all cases. Similarly, negative results were obtained by Calkins (1926, p. 206), for *Uroleptus mobilis*, by Myers (1927) for *P. caudatum*, Grimwald (1928) for *C. colpoda*, and Jahn (1929) for *Euglena*. Among the more recent investigators, Beers (1933) also arrived at negative results for *Didynium nasutum* and *S. pustulata*. In order to prevent any possible modification of the result by bacteria, he employed inorganic solutions as media, and in one case *Paramaecium*, in another *Chlamydomonas*, as food. However, he did not take into account that the allelocatalytic effect is not species-specific (according to Petersen, 1929) and that, consequently, the presence of a relatively large number of food animals in these cultures was bound to preclude the detection of allelocatalysis. Lastly, Phelps (1935), investigating mass cultures of *Glaucoma pyriformis*, free of bacteria, concluded that "experiments on *Glaucoma* do not support the theory of allelocatalysis."

This summary of negative results does not deal with the frequently quoted experiments of Peskett (1924, 1925), as these were carried out on yeast, an object possessed of many biological peculiarities rendering the interpretation of the results extremely difficult.

The third group includes publications that confirm the increased division rate in

cultures with larger inoculum but try to account for this phenomenon by changes in the medium. The first to be mentioned here are the papers adducing the bacteria as the factor determining the observed variations, i.e., those of Cutler and Crump (1924) on *C. colpoda*, that of Chejfec (1929) on *P. caudatum* and of Johnson (1933) on *Oxytricha fallax* and 1936 on *P. caudatum*. They showed to what extent the growth of the cultures is influenced by the bacteria, and concluded that the phenomenon termed "allelocatalysis" is an effect of the variations in the number of bacteria. On the other hand, a theory put forward by Darby (1930) assumes that the ciliates modify the pH of their nutrient medium in optimum direction, thus producing a more favorable reaction in the presence of a higher number of animals per volume.

Briefly summarizing the literature, we must admit that in most cases an allelocatalytic effect was not proved at all, and that such few results as were positive could be explained by accessory circumstances involved by the conduction of the experiment, without necessitating the assumption of an allelocatalytic theory.

When I decided, despite these facts, to undertake the experiments to be described, I was determined by the following reasons. Anyone working on protozoan cultures may easily experience that the success of the culture is, to a high degree, dependent on the size of the inoculum. Cultures with a large amount of initial material will succeed more readily than those with a smaller amount. The latter will frequently grow scantily in the beginning and, after a more or less prolonged interval, start accelerated growth, with a jerk. Similar phenomena may be observed in cultures of algae or bacteria. Lastly, the conditions in tissue cultures, as described by Fischer (1933), should be mentioned.

Moreover, as the material at my disposal allowed of mass culturing, a technique that has been employed in only few allelocatalytic experiments up to the present, I felt that it was worth while, despite the numerous negative results, to investigate the problem once more.

MATERIAL AND METHODS

All experiments were performed on cultures of *Mayorella palestinensis*, a soil amoeba, the cultivation of which is described in a previous paper (Reich, 1935). Great care was exercised to eliminate all disturbing accessories. The series of cultures to be compared were all inoculated into the same nutrient solution, using the same mother culture. The test tubes used in corresponding experiments were all of the same glass (in one part of the experiments, Jena glass; in another part, Pyrex). The diameters were as nearly equal as possible, so as to avoid the disturbing influence of different heights of liquid columns. The tubes were filled with 4.5 cc. of nutrient solution and sterilized in the autoclave. Well-growing young amoeba cultures were centrifuged, washed four times with a 0.1 per cent solution of NaCl, and diluted with the same solution to the requisite concentrations of amoebae. Of the suspension thus prepared, 0.5 cc. per culture were inoculated. Before taking out a sample, part of the liquid was sucked up into the pipette and then powerfully evacuated. This process was repeated several times so as to insure that the amoebae were distributed as uniformly as possible. The eddies thus produced were sufficient to keep afloat such small-sized animals as the amoebae. In view of the smallness of the animals and of the necessity to work under sterile conditions, direct counting of the amoebae had to be dispensed with. Table 1 gives a survey of the result of counting an amoeba suspension and the five cultures inoculated with it. Counting was performed in the Thoma blood-counting chamber. After thoroughly

shaking the cultures, 10 drops of each were counted. As five cultures were made for every grade of dilution, the figures in the tables represent eleven average values of 50 counts and refer to 0.1 cu. mm. of nutrient solution.

TABLE 1

THE RESULT OF COUNTS IN NUMBER OF ANIMALS IN A SUSPENSION OF AMOEBAE AND IN THE CULTURES MADE UP FROM IT

	Number of Amoebae in Each of Ten Counts										Average of Test Tube	Average of the Five Cultures
	1	2	3	4	5	6	7	8	9	10		
Suspension.....	9	9	6	9	8	8	13	4	9	5	8.0	
Culture 1.....	1	1	1	3	1	0	0	0	2	1	1.0	
2.....	0	0	2	0	2	1	0	0	2	0	0.7	
3.....	2	0	1	0	1	1	0	0	1	1	0.7	0.78
4.....	1	0	2	0	1	1	1	1	1	0	0.8	
5.....	1	1	0	0	2	0	0	1	0	2	0.7	

In the experiments with extracts, only 4 cc. of nutrient solution were used and 0.5 cc. of the extract or, in the controls, 0.5 cc. of the extracting liquid were added. The pH of the cultures was registered before and after inoculation and was found to be unaffected. All cultures were kept in a thermostat at 28° C. In view of the slow growth of the cultures it was found sufficient to count them after every 48 hours. Where cysts occurred, counting was given up, as such cultures will not yield any comparable values.

DEVELOPMENT OF THE CULTURES

In order to survey the development of any normal culture, we choose a random example, as represented in Table 2 and Figure 1. Considering first of all the increase in

TABLE 2

THE DEVELOPMENT OF A NORMAL CULTURE

	Number of Days of Culturing										
	0	2	4	6	8	10	12	14	16	18	20
Number of amoebae.....	1.0	1.4	3.9	7.3	9.0	14	19	21	26	26	29
Rate of division per 48 hours.....	.....	0.5	1.5	0.9	0.3	0.7	0.4	0.1	0.3	0.0	0.2
Total number of steps of division.....	.....	0.5	2.0	2.9	3.2	3.9	4.3	4.4	4.7	4.7	4.9

amoeba number, we find that within the first 48 hours after inoculation there is but a slow rise. This is followed by a short period of relatively rapid growth, which gradually passes over into a phase of ever decreasing rate of multiplication. This outline corresponds to the scheme of growth described by Phelps (1935), except that the final stationary phase is not represented in my example. It was omitted since it sets in simul-

taneously with the formation of cysts. During the prolonged subsequent period the number of amoebae fluctuates faintly about a constant value, whereas the number of cysts rises continuously. It is, of course, hard to decide whether the cysts are to be entirely neglected in the definite figuring of the population maximum or to be taken into account, and if so, in what form. This question, being unessential for the problem of allelocatalysis itself, has been left open, and the counts were stopped at the appearance of cysts.

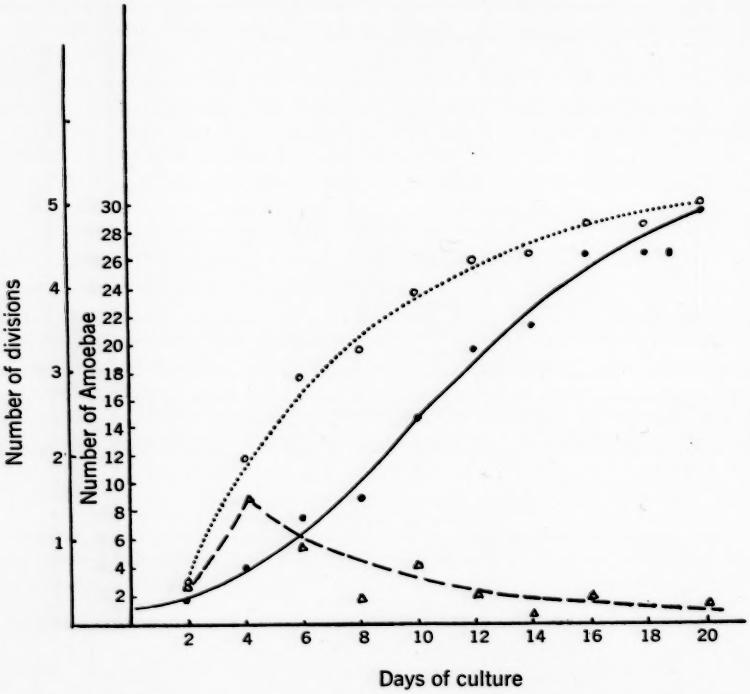

FIG. 1.—Development of an amoeba culture. The number of amoebae, ●——●; the rate of division in each period of 48 hours, △– – –△; the sum of steps of division since the beginning of the culture, ○·····○.

The development of the same culture may be represented in another way. If the rate of division for each particular period of 48 hours is worked out, it is seen that to the slow initial increase in cell number there corresponds a low rate of division, which subsequently rises steeply and then, slowly and gradually, declines. If the total number of steps of division since the beginning of the culture after every 48 hours is worked out and plotted, the curve obtained rises steeply in the beginning and then more and more approaches the horizontal.

After considering which of these modes of representation would be best suited to reveal the presence or absence of allelocatalysis, we selected the last mentioned method.

This offers the advantages, first, of automatically eliminating accidental fluctuations of the individual counts, and, second, of yielding relative values, which are, in the beginning of the curve, independent of the absolute number of amoebae present in the culture. In the absence of allelocatalysis the initial parts of the curves of one experiment will be identical, unaffected by the absolute number of individuals inoculated in the different series of cultures. On the other hand, if allelocatalysis is at work, this part of the curve will be the less steep, the smaller the inoculum employed. The end of the curve is dependent on the number of amoebae in both cases and will be the more nearly level the more nearly a maximum population density will be approached.

EXPERIMENTS ON ALLELOCATALYSIS

The first experiment was conducted in three series (*a*, *b*, *c*,) each of which comprised five test tubes. The results are shown in Table 3 and Figure 2. The series *a* started off

TABLE 3

THE DEVELOPMENT OF THE THREE SERIES (*a*, *b*, AND *c*) OF EXPERIMENT NO. 1

Days of Culturing	Number of Amoebae			Rate of Division per 48 Hours			Total of Steps of Division		
	a	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*
0	1.0	0.7	0.3	.....	.....	.....	.....	.....	.....
2	1.4	1.0	0.4	0.5	0.5	0.4	0.5	0.5	0.4
4	3.9	1.7	0.3	1.5	0.8	.....	2.0	1.3	0.4
6	7.3	4.3	0.5	0.9	1.2	0.4	2.9	2.6	0.8
8	9.0	5.3	0.8	0.3	0.3	0.6	3.2	2.9	1.4
10	14	10	1.7	0.7	1.0	1.0	3.9	3.9	2.4
12	19	14	2.9	0.4	0.5	0.8	4.3	4.4	3.2
14	21	18	5.6	0.1	0.4	0.9	4.4	4.7	4.2
16	26	20	8.2	0.3	0.1	0.5	4.7	4.8	4.7
18	26	26	11	0.0	0.4	0.5	4.7	5.2	5.2

with a concentration of 1 amoeba per 0.1 cu. mm. of nutrient solution. In the first 48 hours the division rate amounted to 0.5; later it rose to a maximum of 1.5, and then decreased slowly and gradually. The maximum population density attained on the eighteenth day of culturing came to 26 amoebae per 0.1 cu. mm. of solution. Series *b* began with 0.7 amoebae per 0.1 cu. mm. of solution. The rate of division lagged at first somewhat behind that of series *a*, but then rose quickly so as to reach the total number of steps of series *a* on the tenth day. The experiment was interrupted on the eighteenth day of culturing, when the numbers of amoebae in the two series were equal. But as the initial number of series *a* exceeded that of series *b*, the total number of steps of division in the latter was higher (5.2) than in the former (4.7). The third series started with a concentration of 0.3 amoebae per 0.1 cu. mm. The rate of division was very low in the beginning, and more vigorous multiplication did not set in before the tenth day. The experiment was broken off before this series had attained its maximum density.

In the second experiment (Table 4 and Fig. 3) the initial concentrations were 0.6, 0.2, and 0.04 amoebae per 0.1 cu. mm. in series *a*, *b*, and *c*, respectively. The development of the cultures resembled that of experiment No. 1. The rate of division in series *a*

was highest at the very beginning and afterward fell off gradually; in series *b* the curve was distinctly less steep in the beginning, and the rate of division reached its maximum

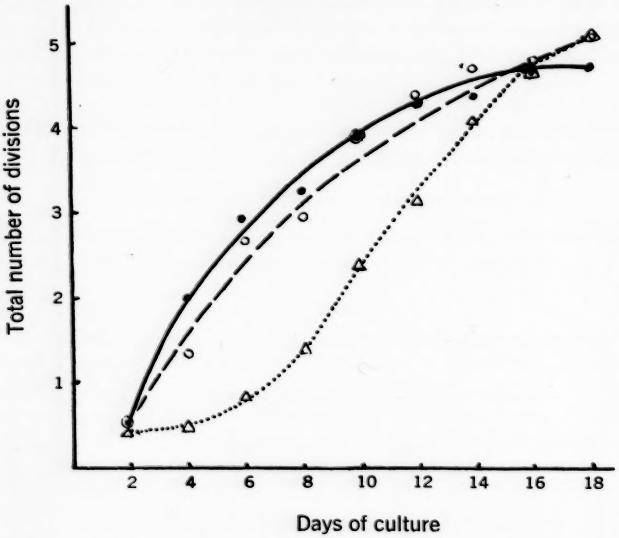

FIG. 2.—The sum of steps of division in the three series of experiment No. 1. Initial populations, given as number of amoebae per 0.1 cu. mm. of medium, as follows: series *a*, ●——●, 1.0; series *b*, O---O, 0.7; series *c*, △·····△, 0.3.

TABLE 4

THE DEVELOPMENT OF THE THREE SERIES (*a*, *b*, AND *c*) OF EXPERIMENT NO. 2

DAYS OF CULTURING	NUMBER OF AMOEBAE			RATE OF DIVISION PER 48 HOURS			TOTAL OF STEPS OF DIVISION		
	a	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*
0	0.6	0.2	0.04	.....	.....	.....	.....	.....	.....
2	1.9	0.4	.....	1.5	0.8	.....	1.5	0.8	.....
4	4.5	0.6	0.08	1.2	0.7	1.0	2.8	1.5	1.0
6	7.8	1.0	0.08	0.8	0.7	0.0	3.6	2.2	1.0
8	12	1.0	0.04	0.6	.....	.....	4.2	2.2	1.0
10	14	2.5	0.16	0.3	1.4	1.0	4.5	3.6	2.0
12	21	5.3	0.08	0.5	1.1	.....	5.0	4.7	2.0
14	.....	10	0.28	.....	0.9	0.8	.....	5.6	2.8
16	.....	14	0.24	.....	0.5	.....	.....	6.1	2.8
18	.....	18	0.28	.....	0.4	0.0	.....	6.5	2.8
20	.....	.....	0.28	.....	.....	0.0	.....	.....	2.8
22	.....	.....	0.64	.....	.....	1.2	.....	.....	4.0
24	.....	.....	1.8	.....	.....	1.5	.....	.....	5.5
26	.....	.....	3.2	.....	.....	0.8	.....	.....	6.3
28	.....	.....	7.1	.....	.....	1.2	.....	.....	7.5

not before the tenth day; while in series *c* the curve was almost level in the beginning, and a more intensive and regular multiplication set in only about the twentieth day.

Experiment No. 3 (Table 5 and Fig. 4) was performed in two series with initial concentrations of 0.4 amoebae in series *a* and 0.04 in series *b*. The results again show, in series *a* maximum multiplication in the first days and slow decrease during the subse-

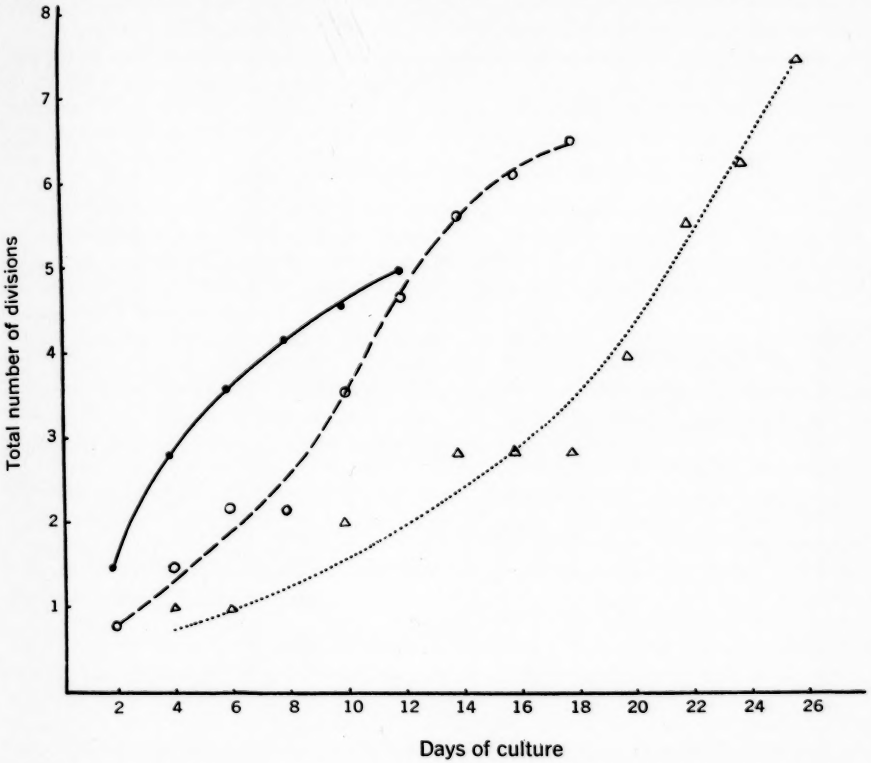

FIG. 3.—The sum of steps of division in the three series of experiment No. 2. Initial populations, given as number of amoebae per 0.1 cu. mm. of medium, as follows: series *a*, ●——●, 0.6; series *b*, ○– – –○, 0.2; series *c*, △····△, 0.04.

quent period, whereas in series *b* intense multiplication starts only toward the end of the time of the experiment.

A fourth experiment with the same concentrations offered a similar aspect, although the rate of division rose somewhat earlier in series *b*, so as to attain its maximum already between the sixteenth and eighteenth days.

Surveying the total result of all the four experiments, we must state the fact that in all cases the series with lower initial concentrations of amoebae showed a much smaller initial rate of division than the cultures with higher initial concentrations under identical

TABLE 5

THE DEVELOPMENT OF THE TWO SERIES (*a* AND *b*) OF EXPERIMENT NO. 3

DAYS OF CULTURING	NUMBER OF AMOEBAE		RATE OF DIVISION PER 48 HOURS		TOTAL OF STEPS OF DIVISION	
	a	*b*	*a*	*b*	*a*	*b*
0	0.4	0.04	.....	.....	.....	.....
2	1.7	0.00	2.1	.....	2.1	.....
4	4.6	0.16	1.4	2.0	3.5	2.0
6	8.1	0.16	0.8	0.0	4.3	2.0
8	14	0.16	0.9	0.0	5.2	2.0
10	16	0.24	0.1	0.6	5.3	2.6
12	.....	0.4	.....	0.7	.....	3.3
14	.....	0.56	.....	0.5	.....	3.8
16	.....	0.96	.....	0.7	.....	4.5
18	.....	1.2	.....	0.3	.....	4.9
20	.....	1.7	.....	0.5	.....	5.4
22	.....	2.0	.....	0.2	.....	5.6
24	.....	3.3	.....	0.7	.....	6.3
26	.....	9.0	.....	1.5	.....	7.8

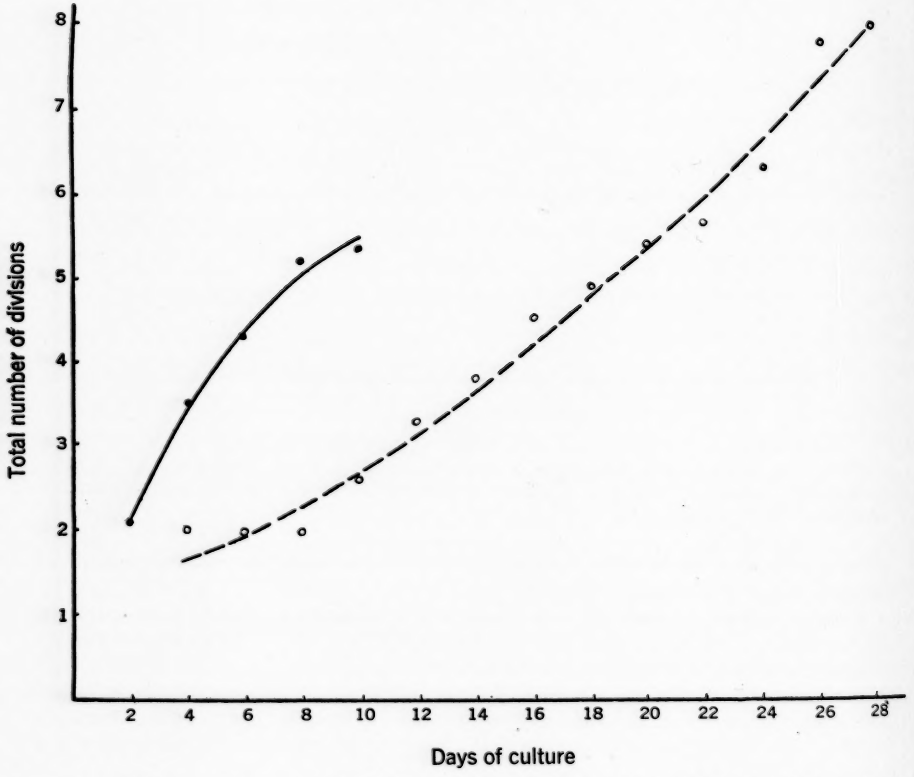

FIG. 4.—The sum of steps of division in the two series of experiment No. 3. Initial populations, given as number of amoebae per 0.1 cu. mm. of medium, as follows: series *a*, ●——●, 0.4; series *b*, ○---○, 0.04.

conditions. While in the highest concentrations the maximum rate of division was attained immediately or soon after inoculation, decreasing slowly with rising concentrations, the series with scanty initial populations showed a small rate of division in the beginning, which increased as the population grew denser, and fell off after a certain maximum density was reached. This phenomenon became fully manifest in those cultures that started off with a population below 0.3 amoebae per 0.1 cu. mm. medium. This behavior confirms the fundamental assumption of the allelocatalytic theory, i.e., that living cells exercise a mutual influence on their multiplicative activity, causing it to rise with increasing population density up to a certain point, after which it remains independent of population number for a time, until it falls off when the optimum density has been exceeded.

EXPERIMENTS WITH VARIOUS EXTRACTS

Robertson's explanation for the allelocatalytic phenomenon refers it to the action of certain substances diffusing from the nuclei into the surrounding medium. If this is correct, it must be possible to increase the division rate by using nutrient solutions in which amoebae have previously multiplied. This assumption was to be checked by a large series of experiments.

The first experiment was carried out with solutions in which amoebae had been kept for various lengths of time. The solutions were prepared by inoculating test tubes, counting daily, and centrifuging when the requisite density of amoebae was reached. The nutrient solution in the control tubes had undergone similar treatment, except that, in place of inoculating by amoeba suspension, it was diluted with NaCl solution. Measured quantities of the supernatant nutrient solution were pipetted off and distributed into sterile test tubes. All tubes were then inoculated with the same culture. This experiment included seven series, in which the nutrient solutions had been centrifuged off at various amoeba densities from 0.9 to 13 individuals per 0.1 cu. mm. solution. The cultures in whose medium amoebae had previously grown did not differ in any appreciable manner from the controls.

In the next experiment four series of tubes were inoculated with different amoeba concentrations so as to produce initial densities of 1.3, 0.4, 0.08, and 0.04 amoebae per 0.1 cu. mm. (0.0 amoebae in the controls). After 24 hours all cultures were centrifuged, the supernatant solutions of each series were separately collected, and measured volumes were transferred to sterile test tubes. Subsequently, the tubes of all series were inoculated with one amoeba culture. As in the previous instance, the new cultures did not exhibit any effect of the former existence of amoebae in the medium.

In view of all those negative results it remained to be seen if the hypothetically active substance might be extracted from the animals themselves. Ten young cultures were centrifuged, and the sediment was then left for 3 hours in 10 cc. of NaCl solution. This extract was added to the nutrient solution and did not prove more effective than the control NaCl solution.

Since with plant auxines chloroform extraction yields particularly good results, this method was also tested. The liquid of fifty cultures was centrifuged off, and 5 cc. of chloroform were added to the sediment. After 48 hours the chloroform was pipetted off and passed through sterile filter paper. By allowing the filtrate to evaporate at room temperature, a solid residue was obtained, which was taken up in 5 cc. of nutrient solution. Of the extract thus prepared, 0.5 cc. was added to every 4 cc. of nutrient solution.

There were two series of controls. (1) The centrifuged liquid medium of twenty-five cultures was shaken up with 5 cc. of chloroform, and the chloroform pipetted off after 48 hours and further treated as above described. (2) Five cubic centimeters of chloroform were filtered and evaporated, and the residue was treated as in the experiment. The cultures developed equally well in all three series without exhibiting the least effect of the extract.

Lastly, an extract was prepared by centrifuging 100–150 cultures, drying the washed amoeba sediment over $CaCl_2$ at room temperature in an desiccator, and extracting with 0.1 per cent NaCl solution for 48 hours. At first these experiments, too, failed to produce any result. However, in the second part of this experiment the nutrient solution employed had been previously autoclaved four times over. Such a solution considerably prolongs the period of inhibited division at the beginning of each culture. Therefore, it was hoped that in such a culture the stimulation of division, if at all existent, should become most clearly manifest. In fact, in two out of seven cultures a marked positive divergence from the controls without extract was observed. However remarkable this effect may be, no conclusive value can be attributed to it. But it may be possible, by the same method with improved technique, to achieve a positive result with certainty in all cases.

DISCUSSION

Surveying the state of the problem once more, we must emphasize, first of all, that cultures containing bacteria will hardly yield conclusive results. The papers quoted above show clearly how the multiplication of the Protozoa in cultures is largely dependent on the bacteria present. Even in those cases where the numbers of bacteria were determined, their possible influence on the Protozoa remained undefined. If it be assumed that the allelocatalytic effect is due to substances given off by the living cell, the bacteria present may act in two possible ways: either they may give off a similar substance themselves, thus masking the effect of variation in protozoan densities, or, on the other hand, they may destroy the substance. In both cases the effect of the allelocatalytic agent will not be demonstrated by the experiment. A similar argument would hold if the effect should be due to rays (like the mitogenetic rays) instead of chemical substances. We should consider, moreover, that experiments with cultures containing bacteria are not to be reproduced without the utmost difficulty and, last but not least, that an exact analysis of the metabolism of the Protozoa which might enable an insight into the phenomenon is hardly possible in such cultures. Petersen (1929) and Johnson (1933) showed that the results vary according to whether small quantities (2 drops) or large volumes (20–40 drops) of nutrient solution are used. It must again be stressed, in this connection, that in the present experiments with *M. palestinensis*, hardly 17 μ in size, the allelocatalytic effect did not appear before there were more than 0.3 cu. mm. medium per individual. We must conclude that in a large percentage of the experiments the volumes of medium used were too small in relation to the volume of the animals present. Furthermore, the use of smallest quantities of nutrient solution involves a number of other sources of error, such as, variability in the pH, change in salt concentrations due to evaporation, and, particularly, accumulation of metabolic products, perhaps combined with scarcity of food.

We are thus left with only the experiments of Phelps (1935), who worked on mass cultures free of bacteria but whose results are directly opposed to mine. I could not

detect the cause of this contradiction in his conduction of the experiments, though possibly the yeast extracts employed may have introduced allelocatalytically active substances into the medium, or the high rate of division of *Glaucoma* may have rapidly obliterated the differences existing in the initial stages, so that they did not find expression in the results.

Although my experiments confirm the allelocatalytic effect as such, they do not contribute to the explanation of the phenomenon. Robertson's hypothesis of discharge of allelocatalytically active substances from the cell during division could only be established if such substances were to be actually detected in old nutrient media or in cell extracts. Before this is achieved, the theory cannot claim higher probability than any other possible explanation. Of these, the mitogenetic radiation may be specially mentioned. As far as known to me, it has not been investigated in this connection, though it is not out of question as a possible cause of the described phenomena.

SUMMARY

The problem of allelocatalysis was investigated in mass cultures of *Mayorella palestinensis*, free of bacteria; and the following results were obtained:

1. The cultures whose initial population was less than 0.3 amoebae per 0.1 cu. mm. of solution showed a distinctly lower rate of division in the first days than the controls with higher initial populations. With increasing density of population, the rate of division in the less densely populated cultures increases, and the maximum population eventually attained is largely independent of the quantity of inoculum.

2. Neither nutrient solutions in which amoebae had been cultured for various lengths of time nor amoeba extracts succeeded in producing any marked increase in division rate.

These results, though confirming the fact of allelocatalysis, cannot afford a support to Robertson's explanation of this phenomenon.

LITERATURE CITED

ALLEE, W. C. 1931. Animal aggregations. Chicago: University of Chicago Press.

BEERS, C. D. 1933. The relation of density of population to rate of reproduction in the ciliates *Didinium nasutum* and *Stylonychia pustulata*. Arch. f. Protistenk., **80**:36.

CALKINS, G. N. 1926. The biology of the Protozoa. Philadelphia: Lea & Febiger. Pp. 623.

CHEJFEC, M. 1929. Die Lebensdauer von *Paramaecium caudatum* in Abhängigkeit von der Nahrungsmenge. Acta biol. exper., **4**:73.

CUTLER, D. W., and CRUMP, L. M. 1923. The rate of reproduction in artificial cultures of *Colpidium colpoda*. II. Biochem. Jour., **17**:875.

———. 1924. The rate of reproduction in artificial cultures of *Colpidium colpoda*. III. *Ibid.*, **18**:903.

———. 1925. The influence of washing on the reproductive rate of *Colpidium colpoda*. *Ibid.*, **19**:450.

DARBY, HUGH H. 1930. Studies on growth acceleration and yeast. Jour. Exper. Biol., **7**:308.

FISCHER, A. 1933. Gewebezüchtung und ihre Beziehung zur allgemeinen Biologie. Zool. Anz., **6**, Suppl., p. 83.

GREENLEAF, W. E. 1926. The influence of volume of culture medium and cell proximity on the rate of reproduction of Infusoria. Jour. Exper. Zoöl., **46**:143.

GRIMWALD, E. 1928. Recherches sur les facteurs du développement des cultures de microorganismes. L'Action de la substance allélocatalitique apparait-elle dans les cultures du *Colpidium colpoda* Ehrb. Acta biol. exper., **3**:81.

JAHN, T. L. 1929. Studies on the physiology of the euglenoid flagellates. I. The relation of the density of population to the growth rate of *Euglena*. Biol. Bull., **57**:81.

JOHNSON, W. H. 1933. Effects of population density on the rate of reproduction in *Oxytricha*. Physiol. Zoöl., **6**:22.

———. 1936. Studies on the nutrition and reproduction of *Paramecium*. *Ibid.*, **9**:1.

MYERS, E. C. 1927. Relation of density of population and certain other factors to survival and reproduction in different biotypes of *Paramecium caudatum*. Jour. Exper. Zoöl., **49**:1.

PESKETT, G. L. 1924. Allelocatalysis and the growth of yeast. Biochem. Jour., **18**:866.

———. 1925. Studies on the growth of yeast. I and II. *Ibid.*, **19**:464 and 474.

PETERSEN, W. A. 1929. The relation of density of population to rate of reproduction in *Paramecium caudatum*. Physiol. Zoöl. **2**:221.

PHELPS, A. 1935. Growth of Protozoa in pure culture. I. Effect upon the growth curve of the age of the inoculum and of the amount of the inoculum. Jour. Exper. Zoöl., **70**:1.

REICH, K. 1935. The cultivation of sterile amoeba on media without solid food. Jour. Exper. Zoöl., **69**:497.

ROBERTSON, T. B. 1921. Experimental studies on cellular multiplication. Biochem. Jour., **15**:595.

———. 1924*a*. Allelocatalytic effect in cultures of *Colpidium* in hay-infusion and in synthetic media. *Ibid.*, **18**:1240.

———. 1924*b*. The influence of washing upon the multiplication of isolated Infusoria and upon allelocatalytic effect in cultures initially containing two Infusoria. Australian Jour. Exper. Biol. and Med. Sci., **1**:151.

———. 1927. On some conditions affecting the viability of cultures of Infusoria and the occurrence of allelocatalysis therein. *Ibid.*, **4**:1.

YOCUM, H. B. 1927. The effect of quantity of culture medium on the division rate of *Oxytricha*. Anat. Rec., **37**:144.